

ANTI-EXOCRINE AUTOIMMUNITY IN THE NOD MOUSE MODEL OF
SJÖGREN'S SYNDROME

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in
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CHARACTERIZATION OF ANTI-EXOCRINE AUTOIMMUNITY IN THE NOD
MOUSE MODEL OF SJÖGREN'S SYNDROME

By

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The primary goal of this dissertation is to elucidate key features of the pathogenesis of anti-exocrine autoimmunity in the NOD (non-obese diabetic) mouse. In addition to autoimmune islet cell destruction, the NOD mouse develops chronic lymphocytic infiltration of the salivary and lacrimal tissues, leading to dramatic declines in exocrine gland secretion. The finding that secretory dysfunction in the NOD mouse correlates to the presence of leukocyte infiltration of the exocrine tissues prompted the study of this strain as a novel model of secondary Sjögren's Syndrome. To further develop this model, the studies presented in this dissertation investigate the anti-exocrine infiltrating cell populations, physiological and biochemical alterations in NOD exocrine tissues, and the contribution of the NOD genetic background to the development of anti-

exocrine autoimmunity. By flow cytometric analysis, the infiltrating lymphocyte populations are shown to be quantitatively and qualitatively similar to that observed in exocrine biopsies from Sjögren's patients and other animal models of this disease. Similarly, RT-PCR detection of cytokine gene expression demonstrates similar cytokine profiles in the NOD exocrine tissues as that seen in human patients and other disease models. The investigation of the NOD-*scid* strain shows that morphological and biochemical alterations of the salivary glands of NOD mice occur in the absence of lymphocytic infiltration, suggesting that intrinsic alterations of the salivary glands may underlie autoimmune invasion. Furthermore, aberrant gene expression and proteolytic processing of the salivary protein, PSP, were detected in both NOD and NOD-*scid* animals. This protein, previously reported as salivary gland specific, is further found to be expressed in the lacrimal glands of mice. The novel cleavage of PSP is the result of a proteolytic activity uniquely present in the saliva of aging NOD and NOD-*scid* animals. Both cysteine protease and matrix metalloproteinase activities were detected in older NOD and NOD-*scid* samples, potentially indicating increased apoptotic activity and glandular restructuring, respectively. Novel proteolytic activity may explain the loss of submandibular acini in NOD-*scid* mice, as well as potential candidate enzymes for PSP cleavage. As such, the findings presented in this dissertation lay the foundation for future genetic and molecular studies investigating the underlying pathogenesis of autoimmune Sicca syndrome.

CHAPTER 1 INTRODUCTION

Sjögren's Syndrome is a progressive, debilitating disorder in which the body's immune system destroys the mucinous secreting exocrine tissues resulting in the hallmark features of dry mouth and dry eyes (sicca syndrome) (1). While classified as an orphaned autoimmune disease, the estimated number of cases of Sjögren's Syndrome in the United States is believed to range between 200,000 to 4 million (2). This wide range in estimated case numbers is attributed to the difficulty in properly diagnosing the seemingly ubiquitous nature of many of the patient complaints. In Europe, where the criteria for diagnosis is less stringent than in the United States, the prevalence is estimated at 0.1 to 1.0% of the population. As typical of many autoimmune diseases, over 90% of Sjögren's Syndrome patients are women, most of which are post-menopausal (2). Common complaints include "trouble chewing or parched mouth," "burning throat," "grit or glass in the eyes," "blurred vision," "itching skin," or "debilitating fatigue"(3). Indeed, Sjögren's has been termed the "great mimicker" since many symptoms, often considered minor or vague, resemble those seen in other disease states such as hepatitis C infection, autonomic neuropathy, or drug treatment (1). Since symptoms are often reported to medical specialists such as dentists or optometrists, Sjögren's Syndrome is often misdiagnosed and symptoms treated as individual entities instead of systemic disorders. These complications in diagnosis have led to the consensus described by the National Organization for Rare

Disorders (NORD) president Abbey Meyers that "Sjögren's Syndrome isn't a rare disease; it's just massively under-diagnosed". The etiology of Sjögren's is unknown, and there are no known cures. It was not until 1933, however, that sufficient interest led to the landmark studies presented to the medical community by Swedish physician, Henrik Sjögren (4).

Henrik Sjögren

Individual case studies of dry mouth or dry eye patients were presented as early as the late 1800's (5). By 1927, Gougerot made the connection between salivary and lacrimal sicca symptoms, and the linkage of these symptoms to arthritic disease was first presented (6,7). Tying these findings together, Henrik Sjögren studied a patient group predominately consisting of women over 40 years of age and displaying keratoconjunctivitis sicca (4). Sjögren noted the appearance of lymphocytic infiltrates in both the salivary and lacrimal tissues, as well as salivary gland swelling. Over 50% of the patients had a history of arthritis. For the next 20 years, little advancement was made beyond these descriptive observations. In 1951, after publishing numerous supplemental studies, Sjögren concluded that the major criteria for diagnosis were keratoconjunctivitis sicca, xerostomia, and polyarthritis (8). While the etiology of disease remained unknown, Sjögren's microscopic and descriptive findings laid the foundation for the diagnostic criteria which is still in use today.

Clinical Presentation and Complications of Sjögren's Syndrome

The protein and mucin-rich secretions derived from the salivary, lacrimal, and other minor exocrine tissues, i.e. labial and hardarian glands, are essential for maintaining the health and integrity of the oral and ocular surfaces. These secretions provide not only the fluid and electrolytes necessary for tissue homeostasis, but also contain several additional classes of protein constituents (9). These constituents include important anti-microbial defense mechanisms against pathogens such as immunoglobulins, iron chelators, and proteases; growth factors important for mucosal tissue maintenance and regeneration, such as epidermal growth factor (EGF), nerve growth factor (NGF), and transforming growth factors (TGF- α and TGF- β); and mucinous lubricating agents. For the most part, both tear and saliva secretions serve similar functions and contain many of the same protein constituents, e.g., EGF, NGF, TGF- α , lactoferrin, lysozyme, and immunoglobulins (9,10). At the same time, however, saliva and/or tear specific secretory proteins, as evidenced by salivary amylase and digestive enzymes, provide for specialized physiological functions of the individual secretory fluids (9).

The clinical presentation of sicca syndrome is associated with the loss of both the fluid and proteinaceous phases of saliva (1). This is demonstrated by the fact that supplemental tear and saliva substitutes lacking the protein constituents of naturally-occurring exocrine fluids are not complete remedies for patient discomfort associated with dryness (11). Similarly, complaints of chronic dryness are often displayed in a portion of autoimmune diabetic patients with normal flow rates (12,13). This indicates that the

protective protein and mucous constituents in addition to fluid saliva are essential for the maintenance of oral and ocular health.

Disruption of exocrine secretion has severe clinical implications. In addition to patient discomfort, corneal damage due to lack of lubricating fluids and chronic ocular infections can lead to blindness in severely affected patients (14). Similarly, loss of protective saliva and hydration provided by mucinous coatings leads to rampant periodontal disease, caries, candidal infection, and cracking and loss of teeth despite rigorous dental treatment regimens (15). Dryness and severe cracking of the tongue in many patients leads to difficulties in speaking, chewing and swallowing most foods. As in many autoimmune diseases, Sjögren's patients often develop a waxing and waning in the severity of symptoms, leading to periods of moderate health followed by chronic episodes (16). To combat dryness, artificial saliva, tears, and ointments can provide symptomatic relief for many patients; however, severe cases are also treated with anti-inflammatory drugs (16).

Numerous systemic complications frequently appear in Sjögren's patients. The most severe of these is a marked increase in the risk (>40 fold) of developing non-Hodgkin's lymphomas in the salivary glands or cervical lymph nodes (17). In 1991, Fox *et al.* demonstrated that salivary gland lymphomas predominately involve a t(14:18) translocation which allows for increased synthesis of the proto-oncogene *bcl-2* (17). Over-expression of *bcl-2* is known to rescue activated lymphocytes from apoptotic death (18).

Other symptoms include chronic fatigue, itchy skin attributed to both dryness and vasculitis, and digestive disorders. Many patients appear to have central nervous system

disorders, which, in addition to chronic fatigue, further adds to the systemic nature of this autoimmune disease (19).

Modern Diagnosis of Sjögren's Syndrome

New advances in our immunologic and molecular understandings have led to an expansion of the criteria determining the diagnosis of Sjögren's Syndrome. Today, Sjögren's syndrome is subdivided into primary and secondary classifications. Primary Sjögren's syndrome is defined as autoimmune sicca syndrome in the absence of other autoimmune conditions (1). Secondary Sjögren's syndrome occurs in the presence of other autoimmune connective tissue diseases, such as rheumatoid arthritis, systemic lupus erythematosus, or scleroderma. The presence of dry eyes is measured through use of the Schirmer's test of tear production, and Rose Bengal corneal-staining dye, once used by Henrik Sjögren, is still used to visualize corneal damage. Salivary gland scintigraphy is used to evaluate saliva flow rates. In 1960, Bloch *et al.* detected the presence of anti-nuclear antibodies in the sera of approximately 70% of Sjögren's patients (20). This finding was further expanded to identify two specific ribonucleoprotein antigens named SS-A/Ro (60 and 52 kDa isoforms) and SS-B/La (47 kDa) (21). Antibodies directed against SS-A and SS-B are present in approximately 90% and 70%, respectively, of sera of Sjögren's syndrome patients (22). Rheumatoid factor is also present in 70% of patients, including those that do not display joint involvement (23). Therefore, presence of these autoantibodies has been used as a critical criteria for diagnostic purposes.

In the United States, the San Francisco criteria is used to diagnose candidate patients displaying sicca syndrome (1). Under this criteria a positive diagnosis includes the detection of lymphocytic infiltration in labial lip biopsies. Unfortunately, different standards for diagnosis in the international community have led to great controversy in regards to the patient populations used in clinical and basic research studies (24,25). In the European (EEC) criteria, for example, examination of labial lip biopsies for presence of lymphocytic infiltration is not essential for diagnosis. Thus, only an estimated 15% of EEC patients would be diagnosed with Sjögren's Syndrome under the San Francisco criteria (25). Since the patient populations are more restrictive in the San Francisco criteria, comparison of international studies remains controversial.

Genetic Factors in Susceptibility

While the initiating environmental triggers of Sjögren's syndrome are unclear, intrinsic genes contributing to disease susceptibility are thought to be critical potentiators in the development of autoimmune disease. Approximately 12% of SS Sjögren's syndrome patients have a relative with this disease, demonstrating a familial aggregation (26). The strongest disease associations appear related to specific alleles of the human leukocyte antigen (HLA) complex or the corresponding major histocompatibility complex (MHC) in murine models (27). These highly polymorphic gene products serve to bind and present peptide epitopes to lymphocytes. A specific allele has unique peptide binding specificities which are dependent upon the polymorphic amino acid composition in the binding/anchoring clefts of the molecule. The subsequent presentation of peptides serves

as a foundation for the development of both tolerance to self tissue antigens and the ability to bind and present novel exogenous peptides to the immune system (28). Therefore, it is theorized that individuals with specific HLA alleles may be predisposed to selected tissue specific autoimmunity based on the capacity of tissue autoantigens to bind in the HLA binding clefts. In support of this theory is the finding that different autoimmune diseases often correlate to the presence of specific HLA alleles.

In Caucasians with primary Sjögren's syndrome, an increased frequency of HLA-B8 is found in ~60% of Sjögren's patients and only 20% in controls (29). Additionally, 83% of primary Sjögren's syndrome patients displayed HLA-DW3 as compared with 24% in control populations (30). Racial distinction in patient populations is also detected, as Japanese patients display higher frequencies of HLA-DRW52 (31). While these findings were generally true for primary Sjögren's patients, secondary Sjögren's syndrome associated with rheumatoid arthritis did not display an association with either HLA-DW3 or HLA-B8, indicating genetic distinction between patient groups (29,30). Similarly, Sjögren's syndrome associated with systemic lupus erythematosus appears related to DR3, DQw2, and C4AQ0 (complement gene mapped to the HLA region) (31). Specific HLA haplotypes are further associated with differences in the levels of anti-Ro antibody production or high autoantibody levels in general. HLA-DW3, for instance, is associated with decreased salivary flow rates and larger focal infiltration among patients (32). Therefore, the presence of particular HLA alleles may not only contribute to disease risk, but also to specific characteristics of an individual patient's disease.

The importance of non-HLA related genes appears to be important as well. A review of familial studies by Goldstein *et al.* suggested the presence of a dominant, non-

HLA linked gene (33). In addition, discordance of Sjögren's Syndrome among identical twins suggests that an environmental trigger(s) plays a critical role as well. The complex polygenic nature of this disease may explain the widely varying complaints and disease severity among patients afflicted with Sjögren's syndrome.

Infiltrating Lymphocytes

One advantage to the clinical study of Sjögren's Syndrome as compared to many other autoimmune diseases is the routine accessibility of tissue biopsies. Focal infiltrates of the salivary glands are predominately CD4⁺ T lymphocytes, with a smaller percentage of CD8⁺ T cells than detected in peripheral tissues (11). Helper T-cells are predominately of a memory phenotype. Although polyclonal activation of B-lymphocytes is a hallmark feature of this disease, only 10-15% of salivary leukocytic infiltrates are comprised of B-lymphocytes. Interestingly, lacrimal gland infiltrates contain nearly double (~30%) the numbers of B-cells as seen in the salivary glands (34). Whether the increased percentage of B lymphocytes within the lacrimal glands is due to increased B-cell proliferation or active recruitment remains unknown. However, detection of CD23 (blast-2 antigen) in the lacrimal infiltrates of Sjögren's Syndrome patients support the possibility of lymphoproliferation.

Diversity of the T-cell receptor (TCR) repertoire is achieved through the random arrangements of the genes encoding the α and β chains. While numerous TCR α chains are available to generate unique VDJ rearrangements, less than thirty β chains are available to compliment the heterodimeric formation of the TCR. Therefore, it was

questioned whether the generation of autoimmune activity against selected tissue antigens involved the preferential usage of specific variable β ($V\beta$) chains which had a biased affinity for tissue antigen epitopes (35). This idea is supported by the expansion of dominant $V\beta$ and $V\alpha$ T-cells specific for defined experimental antigens, such as myelin basic protein (36). In theory, if a biased TCR $V\beta$ gene predominated a given autoimmune disease, specific TCR depletion therapies could be used to slow disease progression without completely immuno-compromising the patient.

In primary Sjögren's patients, a preferential usage of several TCR $V\beta$ genes has been demonstrated in salivary gland biopsies (35,37,38). However, a similar study of lacrimal tissues showed a highly diverse TCR repertoire, providing a potential indication of the involvement of multiple tissue antigens. Unfortunately, the study of T-cell phenotypes in human patients is hampered by the fact that tissue samples are from the late stages of autoimmune progression. Therefore, the identification of the lymphocyte subsets critical for the initiation of disease necessitates the use of animal models.

Cytokines in Sjögren's Syndrome

The immunoregulatory effects of cytokines have stimulated great interest among researchers. Of note, several studies using immunohistochemistry, *in situ* RT-PCR, or RT-PCR methods have shown that production of inflammatory cytokines is not restricted to infiltrating immunological cells. Indeed, the exocrine tissues appear to produce IL-1 and IL-6 which may contribute to inflammation (39,40). Constitutive production of transforming growth factor beta ($TGF\beta$), a strong immunosuppressive factor, is also

found in these tissues (41). Furthermore, production of IFN γ by infiltrating immune cells has been shown to induce the expression of HLA-DR on the surface of salivary gland epithelium (42). Cytokine production is also important in upregulating expression of ICAM-1 and E-selectin on the surface of salivary endothelial cells in Sjögren's patients (43). Together, these findings have led to the current hypothesis that exocrine cells were not merely passive targets of an aggressive immune system, but may be active participants in the autoimmune process. Whether aberrant tissue expression of HLA protein is involved in stimulating or propagating the autoimmune response has yet to be resolved.

In addition to IL-1 and IL-6, high levels of IL-2, IL-10, IL-12, TNF α and IFN γ are detected in Sjögren's biopsies (11). Levels of IL-4 are typically beyond detection. While much emphasis has been placed upon delineating whether T_h1 (cytotoxic response) or T_h2 (humoral) T-helper responses predominate in Sjögren's Syndrome, a clear picture of the complex cytokine interactions is still beyond reach. The presence of IL-10, described to direct the immune response to T_h2 phenotype, in addition to IL-12 and IFN γ , which mediates the opposing T_h1 phenotype, suggests that the distinction of a T_h1 or T_h2 response may not be applicable for this disease (44). Indeed, the cytokine profiles are highly suggestive of a generalized pro-inflammatory process. Nonetheless, potential clinical usage of cytokine-blocking antibodies or cytokine therapy to direct the immune response to a beneficial state is of extreme interest among researchers.

In addition to immunoregulation, cytokines may play a more direct role in tissue destruction. In vitro studies have now shown that IFN γ is toxic to a human salivary gland ductal cell line (HSG) (45). Similarly, TNF α , whose receptor bears homology to the death domain associated with FAS-induced apoptosis, may play a critical role in mediating

both inflammation and apoptotic events (46). Wu *et al.* demonstrated that TNF α , while not able to induce significant death of the HSG line, was able to act synergistically with IFN γ to induce rapid cell death (45). Whether these cytokines directly serve to induce exocrine cell death in the autoimmune state has yet to be determined.

Implications of Viral Expression

Despite extensive research, the initiating triggers for this disease remain a mystery. Discordance between identical twins and the late disease onset suggests that an exogenous agent, such as glandular trauma or viral infection, may potentiate autoaggression in susceptible individuals. Much interest has focused on the potential role of virus in the initiation of Sjögren's Syndrome. Hepatitis C and HIV infection often result in dry eye and mouth syndromes, but do not have the same histological and autoimmune phenotypes of Sjögren's patients (47,48). Transgenic expression of the *tax* gene from the HTLV-1, a human leukemia virus, in mice results in lymphocytic infiltration of the exocrine glands (49). Detectable *tax* gene sequences were evident in 25% of labial salivary gland biopsies of Sjögren's patients (50). Epstein-Barr Virus (EBV) antigens and genomic DNA has been detected in the lacrimal tissue of Sjögren's patients, however high levels in normal biopsies complicates these findings (51). Of particular interest is the finding that the SS-B/La nuclear autoantigen is associated with viral translation and may also be redistributed to the cytoplasm and cell surface following infection (52). Whether the generation of autoantibodies against the Ro and La ribonucleoproteins is potentially due to their association with viral RNAs or molecular mimicry of viral epitopes is

currently a hot topic among researchers. Thus, the potential involvement of viral infection in the initiation or exacerbation of this disease is intriguing; however, researchers have yet to demonstrate a primary etiologic role of virus in Sjögren's Syndrome.

Autoimmune Disruption of Exocrine Secretion

Stimulation of exocrine secretion is under the control of both sympathetic and parasympathetic innervation. Parasympathetic nerves, primarily allowing for the release of watery phase of saliva, are responsible for basal levels of saliva production as well as mechanically stimulated saliva flow, such as when eating (53). Parasympathetic stimulation leads to the release of electrolytes which form the osmotic gradient needed for salivation. Mucin rich saliva secretion is mediated through sympathetic stimulation. Pilocarpine, a muscarinic receptor agonist, is a drug currently used to treat patients through parasympathetic stimulation (54). In our studies, pilocarpine was used to stimulate watery saliva, while the B-adrenergic receptor agonist, isoproterenol, was used to stimulate proteinaceous saliva flow.

Disruption of the naturally occurring secretory process is a frequent consequence of pharmaceutical drugs or the effects of irradiation in anti-cancer therapies (1). While the pathogenic mechanism of exocrine dysfunction in Sjögren's Syndrome is unclear, autoimmune disruption of the secretory process may occur through degeneration of glandular innervation, blockage of receptor stimulation, cellular destruction, or degeneration of the secreting cells. It is generally believed that immune destruction of the exocrine cells is not the primary mechanism for the loss of exocrine secretion (11). This is

in stark contrast to Type 1 insulin dependent diabetes, for instance, where disease onset occurs when over 90% of the insulin secreting β cell mass is eliminated (55). Histological evaluation of exocrine biopsies reveals that only approximately 10% of the tissue area is replaced by lymphocytic infiltration (56). In areas not in direct contact with lymphocytic foci, large areas of seemingly normal tissues are present. Furthermore, the severity of sialadcnitis does not appear correlated with measured loss of glandular secretions. Equally perplexing is the fact that loss of glandular secretion appears inversely related to the presence of anti-ductal cell antibodies in patient sera (57). These findings have led to the current theory that the exocrine glands of Sjögren's patients are "loafing"(11). Chronic destruction of the normal glandular architecture could lead to tissue dedifferentiation and loss of secretory function.

The classical waxing and waning of symptoms in Sjögren's patients suggests that lymphocytes play a direct role in effecting the loss of salivary function. For instance, if glandular innervation was effectively destroyed, abrogation of the immune response would not be expected to restore function. Therefore, it is possible that cytokines and antibodies released by immune components are playing an active role in disrupting neuro-glandular stimulation. Autoantibodies against the Ro and La antigens, while prevalent in Sjögren's patients, have yet to be directly implicated in the loss of exocrine function; although high levels of maternal autoantibody correlates to an increased risk of severe heart defects in children (1).

Animal Models of Sjögren's Syndrome

The inherent scientific and ethical limitations in the study of human subjects has forced basic researchers to turn to the study of animal models which display specific disease traits. Several murine models for Sjögren's Syndrome have been suggested based on the presence of lymphocytic infiltration in the salivary glands, including the NZB/NZW F₁, MRL/lpr, NFS/sld, and TGF β knockout congenic mice (58-61). The TGF β knockout mouse develops extensive systemic autoimmunity including anti-exocrine infiltration and typically dies within one month of birth (61). In 1994, Haneji *et al.* detailed anti-exocrine autoaggression in NFS/sld mice thymectomized at birth, however exocrine dysfunction was not evaluated in this study (60). The NFS/sld mouse was shown to contain a single recessive gene defect in sublingual gland development, indicating that abnormalities in glandular development may contribute to autoimmune tissue targeting.

The NZB/NZW F₁ and the MRL/lpr mice display lymphocytic infiltration of both the lacrimal and salivary glands in addition to lupus-like disorders, and have therefore been studied as models for secondary Sjögren's Syndrome (58,59). Interestingly, the presence of lymphocytes in these tissues and apparent histological changes in the exocrine glands of these mice does not correlate to severe loss of glandular function. Only NZB/NZW mice >4 months of age displayed slightly abnormal Schirmer tests, and none of these strains showed a loss of salivary flow (54). Walcott *et al.* recently described progressive degeneration of glandular innervation in the lacrimal glands of NZB/NZW mice over 6 months of age (62). Similar to that of human disease, focal infiltration of the exocrine tissues in NZB/W and MRL/lpr mice was more prominent in female than male mice,

occupied no more than 30% of tissue sections, and intensity of the infiltration did not correlate to glandular dysfunction (54).

Lymphocytes infiltrating the exocrine tissues of MRL/lpr mice, which have a genetic disruption of Fas-FasLigand-mediated apoptosis, are predominately CD4⁺ T-cells (63). Analysis of TCR V β expression revealed a diverse T-cell repertoire, however, a skewing of the population to V β 8.1.2 and V β 6 was detected (64). Purified CD4⁺, but not CD8⁺ lymphocytes isolated from MRL/lpr salivary glands are able to transfer sialadenitis to immunocompromised CB17-*scid* mice (65). Interestingly, specific depletion of donor V β 8⁺ or V β 6⁺ lymphocytes prior to transfer significantly inhibited sialadenitis in recipient mice.

Both the NZB/NZW F1 and MRL/lpr mice display similar cytokine profiles as those detected in human tissue biopsies (66). Therefore, since these mice do not develop severe functional deficiency, it is unlikely that the cytotoxic effects of hallmark cytokines such as IFN γ are primary effectors of exocrine dysfunction in murine models. Together these findings demonstrate that the mere homing of leukocytes to the exocrine tissues is not sufficient to cause the loss of exocrine function. In 1992, an advance in the search for an animal model for Sjögren's was made by Hu *et al.* in their description of secretory dysfunction in autoimmune NOD (nonobese diabetic) mice (67).

NOD Mouse

First introduced in 1980, the inbred non-obese diabetic (NOD) mouse exhibits a strikingly similar pathology to that of human Type 1 insulin dependent diabetes mellitus

(IDDM) (55). Lymphocytic destruction of the β -cells of the islets of Langerhans results in a loss of blood glucose regulation due to the loss of insulin secretion. Immune infiltration of the pancreas can begin in NOD mice as early as two weeks of age and begins with the appearance of Class II⁺ monocytes and CD8⁺ T lymphocytes (68). Overt diabetes in NOD mice generally begins between 8-12 wks of age and occurs when >90% of the β -cell mass is destroyed. By 30 weeks of age roughly 80% of female and 20% of male mice become diabetic (55). Interestingly, NOD mice kept in specific pathogen free (SPF) colonies develop diabetes more frequently and at an earlier age than those kept in traditional colonies (69). This indicates that, similar to the human condition, environmental factors play a role in the development of autoimmunity in these mice.

The observation of the lymphocytic infiltration in the NOD mouse is not confined to the pancreas, however, but is also observed in the salivary and lacrimal glands of this strain (70). Interestingly, the parotid gland does not develop extensive lymphocytic infiltration. The first appearance of periductal and perivascular lymphocytic infiltration in the submandibular and lacrimal glands begins at 8-10 wks and 10-12 wks of age, respectively (71). By 18 weeks of age, the exocrine glands display focal lymphocytic lesions with an observable disorganization of normal acinar structure. Despite the early appearance of lymphocytic infiltrates within the submandibular and lacrimal glands, loss of saliva flow and tear production does not typically occur until 14-16 wks of age (67). This result was expanded to show that NOD mice lose approximately 90% of their saliva flow and 30% of tear flow between 8 and 20 weeks of age (71). Unlike diabetes in the NOD, both male and female mice develop infiltration of the exocrine tissues at similar rates (70). These findings reveal that the NOD mouse represents the first-described animal

model for the spontaneous autoimmune-induced loss of both saliva flow and tear production, and, as such, is emerging as an excellent model for the study of Sjögren's Syndrome in humans.

Immunogenetics of the NOD Mouse

The NOD mouse arose during the selective breeding of the inbred cataract Shionogi (CTS) strain from outbred ICR mice (55). In 1974, a single female mouse exhibiting polyuria, severe glycosuria, and weight loss was discovered, and, after extensive inbreeding, the NOD strain was introduced in 1980. Since this time, at least 13 genetic loci have been discovered which contribute to the development of diabetes in NOD mice (72). An additional two genetic loci, *lidd-7* and *lidd-8* found in the B10 background, are also able to increase diabetes progression when bred onto the NOD background (69). Genetic influence contributes to both the incidence of diabetes, as in *lidd-1*, as well the timing of disease onset, as in *lidd-2* and *lidd-4*.

The MHC-linked locus, *lidd-1*, is essential for the development of diabetes, as replacement of the of the NOD MHC loci with the B10-derived allele completely inhibits both diabetes and insulinitis, but not sialadenitis (73). The NOD MHC, H-2^{s7} haplotype contains several unique features, including a structurally distinct I-A molecule and a deletion of I-E expression (74). Conserved serine-aspartic acid or proline-aspartic acid at the A β amino acid positions 56 and 57 are replaced with histidine and serine in the NOD allele (75). In humans, replacement of the aspartic acid with uncharged amino acids at position 57 of the human DQ β has been implicated in increased diabetes risk (76).

Transgenic expression of I-A^k in NOD mice inhibits insulinitis and diabetes, however sialadenitis was not affected (77). The lack of I-E expression is not unique to the NOD strain, but is also a feature of inbred C57BL/6, SJL, ACA, and DBA/1 mice (78). Since the B10 mice used in generating the NOD.B10-H-2^b congenic are also I-E negative, the impact of I-E expression on the NOD strains was not investigated in this model. Generation of transgenic NOD mice which express the I-E molecule has determined that I-E expression is protective against diabetes and insulinitis (79). Sialadenitis was not investigated in these mice. Interestingly, Faustman found occasional lymphocytic infiltration in the submandibular glands in the majority of the I-E negative strains mentioned above (78). The I-E molecule is responsible for the deletion of TCR $\nu\beta 5^+$ T-lymphocytes, suggesting that $\nu\beta 5^+$ lymphocytes may play a role in the development of sialadenitis. However, NOD mice backcrossed with SWR mice, which contain a deletion in $\nu\beta 5$, $\nu\beta 8$ and $\nu\beta 11$ gene segments, still develop typical frequencies of insulinitis and diabetes (80). Together these findings demonstrate that both the unique NOD I-Ag7 molecule and the lack of I-E expression are important for diabetes, however exocrine gland infiltration is only associated with the lack of I-E expression.

The contributions of non-MHC genes to the development of diabetes and sialadenitis is an area of intense interest. Garchon *et al.* linked a centromeric locus on chromosome I with diabetes susceptibility (81). Interestingly, a second susceptibility locus on the same chromosome was linked to sialadenitis, peri-insulinitis, and hyper-IgG production. The development of sialadenitis was found to be a dominant trait in these studies (81). To evaluate immune and non-immune components of autoimmunity in the NOD mouse, Leiter *et al.* bred the *scid* mutation onto the NOD genetic background,

creating the NOD-*scid* strain which was extensively used in the current studies (82). Homozygosity at the *scid* locus leads to the loss of functional T and B lymphocytes, and, therefore, these animals do not develop insulinitis, sialadenitis, or diabetes. This study also demonstrated that, similar to parental NOD mice, NOD-*scid* mice have complement deficiency, functionally immature macrophage populations, and decreased natural killer cell activity (82). The sum results of these immunogenetic findings are promising in that they strongly suggest that the ultimate goal of genetically separating diabetes from exocrine gland autoimmunity to develop a primary murine model for Sjögren's Syndrome will be attainable.

Role of Helper T-Lymphocytes

Prior to this study, the lymphocyte phenotypes infiltrating the exocrine glands were largely unexplored. Therefore, our knowledge of autoimmunity in the NOD mouse comes almost exclusively from diabetes research. While autoimmune infiltration includes several immune cell types, researchers have predominately focused on the role of CD4⁺ T lymphocytes which represent the largest component of the infiltrate. Neonatal thymectomy of NOD mice generally inhibits insulinitis and diabetes, and athymic nude NOD mice do not develop either phenotype (83,84). Similarly, injection of anti-Thy1.2, anti-CD4, or anti-CD3 antibodies suppresses diabetes (55). A direct role of lymphocytic involvement is demonstrated by the ability of splenic or tissue infiltrating lymphocytes from diabetic donors to rapidly transfer both diabetes and sialadenitis to non-diabetic NOD or NOD-*scid* recipients (85). As opposed to rapid transfer of disease, splenic

lymphocyte preparations from prediabetic mice suppressed disease onset. Therefore, the ability to successfully transfer disease is not only dependent upon the presence of CD4⁺ T lymphocytes, but also their activation state.

When transferred to immunocompromised recipients, purified naive CD4⁺ T cells, represented by the CD4⁺CD45RB^{hi} phenotype, induce a wasting disease associated with intestinal inflammation (86). Interestingly, co-transfer of CD4⁺CD45RB^{lo} memory T cells suppresses the wasting disease (87). Two distinct populations of CD4⁺ memory T cells with opposing diabetogenic potential have been separated from prediabetic and diabetic NOD mice based upon their cytokine expressions (87). Memory cells producing high levels of IFN γ (T_H1) and low levels of IL-4 transfer diabetes rapidly to recipient mice. However, memory cells producing low amounts of IFN γ and high levels of IL-4 (T_H2) confer active suppression (87). This indicates that the ability to progress from a protective to a pathogenic state may lie in the relative levels of cytokines present in the autoimmune lesion. This is supported by similar findings using diabetogenic CD4⁺ T-cell clones. Haskins *et al.* demonstrated that shifting high IFN γ producing T cell clones to IL-4 producers in long term culture suppressed their ability to transfer disease (88). Furthermore, successful transfer of diabetes with CD4⁺ T cell clones into NOD-*scid* recipients demonstrates the potential of CD4⁺ cells to transfer diabetes in the absence of either CD8⁺ T cells or B lymphocytes.

Analysis of TCR V β usage in the pancreas of NOD mice shows heterogeneous TCR populations. However, much like the MRL/lpr mouse, a preferential usage of V β 8 and V β 6 genes is present in diabetogenic T cells (89). Edouard *et al.* showed that selective deletion of V β 6⁺ or V β 8⁺ T cells prior to adoptive transfer significantly lowered

the ability to transfer diabetes to irradiated NOD recipients (90). Although suggestive of a pathogenic role for $V\beta 6^+$ and $V\beta 8^+$ T cells, NOD X SWR mice develop insulinitis and diabetes despite deletions in $V\beta 5$, $V\beta 8$, and $V\beta 11$ gene segments (80). In addition, TCR usage from islet specific T cell clones of diabetic NOD mice include $V\beta 4$, $V\beta 6$, $V\beta 8.2$, $V\beta 12$, $V\beta 16$, and $V\beta 19$ gene usage, further indicating that glandular specific TCR gene usage is not restricted (55). Recent findings by Anderson (personal communication) have indicated that TCR $V\beta$ restriction may be present in a $CD3^+CD4^+CD8^-$ "double negative" T cell population which is present in early islet infiltrates; however, this has yet to be confirmed.

CD8⁺ T Lymphocytes and B Lymphocytes

While the necessity of $CD4^+$ lymphocytes is readily apparent, the role of $CD8^+$ T cells and B cells in the initiation of autoimmunity is less clear. Transfer of purified $CD8^+$ T cells is typically insufficient to cause diabetes, however a recent report demonstrated that diabetes can be transferred by a $CD8^+$ T cell clone (85,91). Using a gene knockout strategy, Wicker *et al.* determined that disruption of the β -2 microglobulin gene, which results in a specific loss of $CD8^+$ T-cells, inhibits both insulinitis and diabetes in NOD mice (92). However, in prediabetic mice, co-transfer of both $CD4^+$ and $CD8^+$ populations is necessary to initiate diabetes (85). Therefore, while an aggressive $CD4^+$ or $CD8^+$ T cell clone may be able to cause diabetes, both cell types appear essential for the initiation of disease.

Similarly, despite the fact that autoantibodies are detected on the surface of islet cells prior to leukocytic infiltration, the role of B-cells has been largely downplayed in the literature (93). Renewed interest in the B cell component is being generated with the discovery that NOD mice containing a μ -heavy chain knockout which halts the generation of mature B lymphocytes do not develop insulinitis or diabetes (94). While exocrine dysfunction has yet to be evaluated in these mice, several studies have indicated that the B cell component may be important for exocrine dysfunction in NOD mice. In addition to the presence of antibodies directed against islet cell components, NOD sera contains both anti-nuclear antibodies and antibodies directed against salivary gland cells (95,96). In an analysis of the signal transduction pathways responsible for secretion from salivary and lacrimal glands, Humphreys-Beher *et al.* identified a subclass of autoantibodies directed against the muscarinic and β -adrenergic cell surface receptors that initiate this process (97,98). The density of these cell surface receptors is reduced on both the parotid and submandibular glands, along with secretory response to β -adrenergic, muscarinic/cholinergic and neuropeptide agonists during disease. Thus, it is possible that soluble factors such as autoantibodies may ultimately be responsible for the loss of secretory function in NOD mice. This may also explain how parotid gland function is inhibited despite the lack of focal lymphocytic infiltration. Together, these findings suggest that the initiation of autoimmunity in the NOD mouse involves multiple immune components and numerous cell types.

Potential Dichotomy of Diabetes and Sialoadenitis

Approximately 30% of autoimmune diabetic patients suffer concomitantly from xerostomia and xerophthalmia due to insufficient glandular secretions (13). These clinical symptoms are thought to result from poorly maintained blood glucose regulation, lack of insulin secretion, or neuropathy (99). Expansion of the autoimmune processes into the exocrine glands of diabetic patients is rarely encountered and generally not considered to be the causative factor for exocrine dysfunction in diabetics (12). In support of this, tight glucose control generally alleviates the feeling of dryness in most patients. Therefore, is it possible that the loss of exocrine secretion in NOD mice is due to the loss of blood glucose regulation, and not anti-exocrine autoaggression?

In addition to the immunogenetic studies discussed above, several studies have illustrated a dichotomy between autoimmune diabetes and autoimmune sialadenitis in the NOD mouse. Loss of secretory function in NOD mice, while more severe after diabetes onset, is present in both male and female prediabetic mice, which lose 50-70% of stimulated salivary flow between 8 and 20 weeks of age (67). Similarly, injection of diabetic mice with daily insulin injections does not restore secretory function. Recent findings using NOD.B10-H-2^b mice have shown that the loss of secretory function occurs in the absence of either insulinitis or diabetes (unpublished observations). Treatment of NOD mice with antibodies against alpha 4-integrin and L-selectin was able to significantly inhibit both insulinitis and diabetes, however, sialadenitis was unaffected (100). In humans, increased expression of ICAM-1 and E-selectin is detected on salivary epithelial cells (43). From the standpoint of tissue tolerance, intrathymic injection of islet cell homogenates or

transgenic expression of proinsulin II in NOD mice prevents diabetes but fails to protect against sialadenitis (101,102). These studies suggest that the antigens involved in the anti-exocrine response are distinct from those in the islet, and that loss of exocrine gland tolerance is not secondary to β cell autoimmunity.

Changes in Saliva Proteins in the NOD mouse

In addition to loss of secretory function in NOD mice, the protein constituents of NOD saliva change over time. This is reflected by reductions in amylase activity (>50%) and loss of ductal cell secretion of EGF (67). In newly diabetic male NOD mice, over 97% of EGF production is lost. In this dissertation, specific changes in both parotid secretory protein (PSP) and proline rich proteins (PRP) are observed in both NOD and NOD-*scid* animals which parallel changes in EGF and amylase. The proline rich proteins are latent in normal mice; however, they comprise >70% of the protein in human saliva, where they serve to bind both calcium and hydroxyapatite (103). Expression of the PRPs can be induced in both the murine parotid and submandibular glands by chronic β -adrenergic stimulation or the introduction of high levels of tannic acid in the diet (104). In these studies we demonstrate the abnormal presence of PRPs in both submandibular and parotid glands of aging NOD mice.

In normal mice, PSP is described as a 20 kDa, leucine-rich glycoprotein (~23% leucine; 235 amino acids) of unknown function that is secreted predominately by amylase producing acinar cells of the parotid gland (105). Studies have shown a developmental coordination of murine PSP and salivary amylase expression in the parotid gland of adult

mice where the two proteins appear in constant ratios (106). Coordinate expression, however, is not determined by the rate of gene expression, since developmental expression of the PSP gene occurs before amylase expression (107). Although PSP is specific to the parotid and sublingual glands of adult mice, PSP is expressed in the developing submandibular gland up to 5 days of age (108). This is important in that abnormal re-expression of PSP in the submandibular gland is detected in aging NOD mice and may indicate dedifferentiation of the glandular acini. While the function of PSP is unknown, our findings suggest that it may play a role in anti-microbial binding. To date, PSP has not been detected in human saliva (our anti-mouse PSP antibody does not crossreact), however a single copy of the PSP gene is present in human cells and PSP mRNA has been detected in human parotid tumors (109).

Results in this dissertation show that PSP is enzymatically cleaved in older NOD saliva. The cleavage occurs between leucine and asparagine at the 26th and 27th amino acids of the protein. Database searches uncovered no known enzymes which may be responsible for this cleavage. However, since PSP cleavage corresponds to the time of dramatic acinar cell loss in the NOD mouse, we investigated the potential of both matrix metalloproteinases and apoptotic cysteine proteases to cleave PSP through bystander activity. These proteins, involved in glandular restructuring and apoptotic cell death, respectively, may play a key role in the loss of submandibular acini in NOD and NOD-*scid* mice.

Matrix Metalloproteinases and Cysteine Proteases

Cellular homeostasis depends on regulated cell proliferation coupled to cell death (110). The matrix metalloproteinases (MMPs) are a class of zinc-dependent enzymes which, in conjunction with their specific inhibitors (TIMPS), are responsible for restructuring and maintenance of the extracellular matrix. Over 12 MMPs have been described and include the collagenases, gelatinases (collagen type IV), stromelysins (laminases), and elastins which are able to cleave virtually all components of the extracellular matrix (111). Expression of specific MMPs during the developmental process is responsible for sculpturing the extracellular environment and thereby dictating cellular turnover and differentiation. While much research has focused on their association with tissue homeostasis and tumor metastasis, a prominent role of MMPs in autoimmune processes has recently been described (111). Monocyte production of MMPs is involved in dictating the extent of tissue damage caused by inflammation and may additionally be critical for matrix degradation necessary for lymphocyte chemotaxis. Inhibitors of MMPs have been shown to significantly reduce the appearance and severity of EAE in animal models (112). This effect was attributed to the inhibition of monocyte-derived gelatinases responsible for the degradation of the blood-brain barrier basement membrane. Similarly, MMP inhibitors significantly reduce joint inflammation and tissue destruction in collagen-induced murine model of rheumatoid arthritis (113). Studies have further demonstrated that MMP activity is connected to the apoptotic pathway through the ability to cleave cell surface FAS on apoptotic target cells (111). In humans, Sjögren's patients have elevated levels of collagenase (MMP-1) in their saliva, and supernatants from excised biopsies

contain elevated MMP activities (114). Whether elevated or aberrant MMP production precedes or is a result of tissue inflammation in autoimmune target tissues is still unknown, however the extensive changes in glandular architecture seen in tissue biopsies may, in part, be attributed to MMP activity.

Extracellular signaling molecules are capable of regulating glandular cell populations through a series of intracellular events termed programmed cell death or apoptosis (110). Apoptosis is distinct from cell lysis in that the target cell actively and efficiently mediates its own death. This process involves intracellular activation, reduction of cell volume, chromatin condensation, and endonuclease cleavage of DNA within the cell membrane (115). By this process, the apoptotic cell contents are kept safely inside the cell membrane until phagocytized by macrophages or neighboring cells. Immune system molecules capable of mediating apoptosis include the cytokine tumor necrosis factor (TNF) and the related protein CD95 (FAS) (115). Mice that lack functional FAS surface protein develop a lymphoproliferative disorder and lupus-like pathology associated with the inability to delete lymphocytes during the immunological education process (116). In Sjögren's patients, it is hypothesized that dysfunction in apoptosis results in abnormal longevity of both T and B lymphocytes and may underlie polyclonal B-cell activation. Elevated levels of bcl-2, which is able to rescue target cells from apoptotic death, are detected in salivary gland biopsies (117). Interestingly, 2-fold increases in surface FAS expression are detected in peripheral blood T cells from both Sjögren's Syndrome and SLE patients than controls which correlated with accelerated apoptosis of these cells in vitro (118). Therefore, it is unlikely that defective FAS molecule is directly responsible for Sjögren's Syndrome or SLE in most diseased patients.

Activation of the FAS or TNF receptors leads to a proteolytic cascade involving members of the cysteine protease family (110). These proteases cleave important cellular proteins including pro-enzymes of other members of this class of proteases and endonucleases responsible for DNA cleavage. A prototype protein of the cysteine proteases is interleukin-1 β -converting enzyme (ICE) which has been characterized as the activator of the cytokine interleukin-1 β through cleavage of its precursor at Asp116/Ala117 (119). All members of the ICE-related cysteine protease family, including Nedd-2 and apopain/cpp32, cleave their substrates after an aspartate residue followed by a small amino acid residue which is important for substrate consensus recognition (120). Expression of ICE in neuroblastoma or fibroblast cell lines leads to apoptotic death (110). Therefore, high levels of cysteine protease activity may be indicative of both the activation of apoptotic mechanisms as well as the processing of proinflammatory cytokine precursors. Since both MMPs and cysteine proteases appear involved in regulated cell proliferation and cell death, it is possible that upregulation of these enzymes in the NOD mouse are directly involved in acinar cell death and the aberrant cleavage of additional salivary proteins.

Further Development of the NOD Mouse Model of Sjögren's Syndrome

The physiological loss of secretory function makes the NOD mouse an ideal animal model for the study of human Sjögren's Syndrome. Despite the limited amount of exocrine gland research, the most exciting aspect of the NOD mouse is the ability to apply

the findings of over 15 years of diabetes research to the understanding of autoimmune sicca syndrome. Additionally, the seeming dichotomy between sialadenitis and diabetes suggests that the study of congenic NOD mice, such as the NOD.B10H-2^b, will lead to the development of a primary model to study anti-exocrine autoimmunity. To begin these studies, however, it is necessary to determine the immunological and physiological processes underlying exocrine dysfunction in the NOD mice. Therefore, the studies of this dissertation explore the following specific aims:

1. To determine the temporal changes in exocrine gland histology, infiltrating lymphocyte populations, and cytokine production spanning the initiation of the autoimmune process to the final stages of glandular destruction.
2. To investigate exocrine gland abnormalities in immunodeficient NOD-*scid* mice in order to separate immune and non-immune components of the disease process.
3. To investigate the tissue specificity and function of parotid secretory protein, which is found to be aberrantly expressed and processed in aging NOD mice.
4. To evaluate the proteolytic activity in NOD saliva responsible for the aberrant cleavage of PSP and investigate candidate enzymes for PSP cleavage.

CHAPTER 2

CHARACTERIZATION OF THE CHANGING LYMPHOCYTE POPULATIONS AND CYTOKINE EXPRESSION IN THE EXOCRINE TISSUES OF AUTOIMMUNE NOD MICE

Introduction

During the past several years significant interest has developed in detailing the autoimmune destruction of the salivary and lacrimal tissues in the NOD mouse. Pioncering studies have demonstrated that the lymphocytic infiltration of the salivary and lacrimal glands correlates with a functional decline in saliva flow and tear production independent of the loss of blood glucose regulation observed in NOD mice (67,71). Lymphocyte transfer studies have now shown that the induction of thymic tolerance to the pancreatic islets does not confer immunologic tolerance to the salivary tissues, suggesting a potential dichotomy of disease states between these target tissues (101). This is supported, as well, by immunogenetic studies linking sialoadenitis, hyper-IgG production, and peri-insulitis to a centromeric loci on Chromosome 1, while insulitis is linked to a telomeric loci on the same chromosome (81). In addition to anti-pancreatic and anti-insulin autoantibodies, NOD sera has been shown to contain autoantibodies targeting the acinar and ductal cells of the submandibular and parotid glands, including those directed against the muscarinic and β -adrenergic receptors responsible for the generation of saliva flow (97,98). The decline in saliva output is accompanied by changes in salivary protein content as well. Both alterations appear to be the result of down-regulation of signal

transduction components of the salivary glands (97). Abnormalities in gene transcription and protein processing in the salivary glands of immunodeficient NOD-*scid* mice suggest that glandular dysfunction in NOD mice may precede lymphocytic infiltration and provide a potential trigger for the autoimmune targeting of the salivary glands (121).

The NOD mouse represents the first-described animal model for the spontaneous autoimmune-induced loss of both saliva flow and tear production, and, as such, is emerging as an excellent model for the study of Sjögren's Syndrome in humans. Sjögren's Syndrome is an autoimmune disease characterized by dry eye and dry mouth syndromes due to the destruction of exocrine tissue. Clinical patients develop chronic lymphocytic infiltration of the salivary and lacrimal glands as well as a cell-mediated and autoantibody response against the exocrine tissue (11). Diagnosis of Sjögren's Syndrome often relies on the detection of lymphocytic infiltration in labial lip biopsies excised from patients, which have revealed a predominance of CD4⁺ T lymphocytes with an oligoclonality of the T-cell receptor repertoire (38). In a majority of patients, autoantibodies to ribonuclear protein antigens are also present (20). In addition, increases in IFN γ , TNF α , IL-2, IL-6, and IL-10 cytokine production have been described in biopsy tissues (11). However, since patient samples are from a late stage, the initial events surrounding the development of autoimmune infiltration and destruction of tissue remain unknown.

In this study I have characterized the infiltrating lymphocyte repertoire and cytokine mRNA profile of the lacrimal, parotid, and submandibular glands of NOD mice during the course of immunopathogenesis. Spleen and islet-infiltrating cells were analyzed as control lymphocyte populations throughout the time course of disease progression. This has allowed for a qualitative comparison of lymphocyte populations infiltrating the

pancreas, submandibular and lacrimal tissues of NOD mice, as well as provide a detailed account of the anti-exocrine autoimmune response which can be compared to that seen in Sjögren's Syndrome.

Materials and Methods

Animals

Female NOD mice (7 wks of age) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained throughout the course of the study in the animal facility at the Health Science Center at the University of Florida (Gainesville, FL). Diabetes was diagnosed by elevated blood glucose levels using Chemstrip bG reagent strips (Boehringer Mannheim, Indianapolis, IN). Mice with blood glucose >200 mg/dL were given insulin injections (1U/mouse/day ip) (67). Evidence of diabetes was first noted at 14 weeks of age. At 16 weeks, 3 of 5 mice were diabetic; at 18 weeks, 1 of 5, and at 20 weeks, 2 of 5 mice were diabetic.

Antibodies

Monoclonal antibodies used in this study were purchased from PharMingen (San Diego, CA) and are as follows; CD3 ϵ (clone 145-2c11), CD4 (RM4-5), CD8 α (53-6.7), CD45RB/B220 (RA3-6B2), CD45RB (23G2), TCR V β 3 (KJ25), TCR V β 6 (RR4-7), TCR V β 8.1, 8.2 (MR5-2), TCR V β 9 (MR10-2), TCR V β 11 (RR3-15), and TCR V β 17a (KJ-23).

Tissue Preparation

Spleen, pancreas, lacrimal, parotid and submandibular glands were removed at each harvest. A small piece was cut from each tissue, placed in 10% buffered formalin and submitted to the Diagnostic Referral Laboratory at the University of Florida (Gainesville, FL) for histologic sectioning and staining. The remainder was processed as a pool from the 5 mice per group. A small aliquot of each pool was removed for RNA isolation and all remaining tissue prepared for flow cytometric analyses.

Single cell suspensions of splenic leukocytes were obtained by gently pressing spleens through wire mesh screens and washing in PBS (68). Red blood cells were lysed with 0.84% ammonium chloride. After washing, the remaining leukocytes were aliquoted at 1×10^6 /tube and washed with FACS buffer (PBS with 0.1% NaN_3 (FISHER Scientific, Orlando, FL) and 0.5% BSA (SIGMA Chemical Co, St. Louis, MO) prior to antibody staining.

Tissue Digestion

Except for spleen, all pooled tissues were dissociated by gentle mincing followed by digestion at 37°C in a shaking water bath for 15 minutes in a mixture of 4 mg/ml collagenase Type V (SIGMA) + 100 U/ml DNase Type II (SIGMA). Digested tissue was further dissociated with vigorous pipetting, removed (after allowing large, undigested pieces to settle) and placed in ice-cold HBSS with 2% FBS (GIBCO/BRL, Grand Island, NY). Digestion was continued using a mixture of 2 mg/ml collagenase + 100 U/ml DNase in 5 min incubations at 37°C in a shaking water bath and repeated until completion.

Table 1. Murine primer and probe sequences

	5' primer	3' primer	Internal probe
mIL-1 β	5'- TGGCAACTGTTCTCTGAACCTCACT	5'- CAGGACAGGTATAGATCTCTTCTTT	5'- CCGTGGACCTTCCAGGATGAGGACATGAGC
mIL-2	5'- ATGTACAGCATGGAGCTCGCATC	5'- GGCTTGTGATGATGATGTTTGACA	5'- GCCACAGAAITGAAAGATCTTCAGTGGCTA
mIL-4	5'- ATGGGTCTCAACCCACAGCTAGT	5'- GCTCTTTAGGCTTTCAGGAAGTC	5'- AGGGCTTCCAAGGTGCTTCGCATATTTAT
mIL-5	5'- ATGAGAAGGATGCTTCTGACTTGA	5'- GTCACCATGGAGGAGCTCAGCC	5'- ATAAAAATCACCAAGCTATGCATTGGAGAAA
mIL-6	5'- ATGAAGTTCCTCTCTGCAAGAGCT	5'- CACTAGGTTTGGCGAGTAGATCTC	5'- TCTGGTCTCTGGAGTACCATAGCTACCTG
mIL-7	5'- ATGTCCATGTTCTTTTAGATA	5'- GAAACATGATCATCTCTTTTCT	5'- GAAGCTGCTTTTAAATCGTGTGCTCGC
mIL-10	5'- GCAGGGGCGAGTACAGCCGGAA	5'- GCTTTTCATTTTGATCATCATGT	5'- TCAGGATGCGGCTGAGGCGCTGTCATCGAT
mIL-12 (p40)	5'- ATGTGGGAGCTGGAGAAAGAGG	5'- TCACGACGGGGTGCTGAAGGCGTG	5'- ACTCCGGACGGTTCAAGTGTCTATGGC
mIFN γ	5'- TGAACGCTACACACTGCATCTTGG	5'- CGACTCTCTTTTCGGCTTCTCTGAG	5'- GACATGAAATCTCTGCAGAGCCAGATTATC
mTNF α	5'- ATGAGCACAGAAAGCATGATCCGC	5'- CCAAGTACAGCTGCCCGGACTC	5'- CTCCTGGCCCAACGGGATGGATCTCAAAGAC
mINOS	5'- CCCTCCGGAAGTTTCTGGCAGCAGC	5'- GGCTGTGACAGCCTCTGGGCTTTGG	5'- TGCCGGCACCCGGAGGAGAC
G3PDH	5'- TGAAGGTCGGTGTGAACGGATTGGC	5'- CATGTAGGCCATGAGGTCCACCAC	5'- CCTTCGGTGTCTCTACCCCAATGTGTCCG

Islets were picked manually from digested pancreatic tissue under a dissecting microscope and collected in ice-cold IMDM (GIBCO/BRL) with 1% NMS (68). Islets were collected by centrifugation, supernatant discarded and the cells dispersed by continuous pipetting in 10 ml of a solution containing trypsin/EDTA (GIBCO/BRL) + 2000 U DNase (SIGMA) at 37°C for 10 min. Digestion was stopped by adding ice-cold IMDM with 1% NMS.

Digested lacrimal, parotid and submandibular tissue and dispersed islets were washed and then separated by centrifugation through a 55% Percoll (SIGMA) gradient. The infiltrating cells were collected in the pellet and contaminating red blood cells lysed with 0.84% ammonium chloride. After washing, cells were divided into aliquots containing approximately 1×10^6 cells and washed in FACS buffer prior to antibody staining.

Flow Cytometry

Aliquots of all cell populations were resuspended in 100 μ l FACS buffer and stained with antibody at 1 μ g/ 10^6 cells. Cells were stained first with anti-CD3 for 40 min at 4°C, washed with FACS buffer and then stained with the appropriate second antibody in a 40-min incubation at 4°C. After a final wash, cells were suspended in FACS buffer for analysis.

Flow cytometric analyses were performed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 milliwatt, 488nm air-cooled argon-ion laser and using LYSYSTM 11 software (68). Ten thousand events were collected

pcr sample from a population gated on a window encompassing the splenic lymphocyte population.

RNA Isolation and RT-PCR Detection of Cytokine mRNA

Pooled tissues were minced in PBS, placed in lysis buffer and mRNA isolated using a Micro-FastTrack™ Kit (Invitrogen). Isolated mRNA was stored at -70°C in ethanol until all samples were collected. mRNA was pelleted by centrifugation and cDNA prepared by reverse transcription using Superscript II™ Reverse Transcriptase (GIBCO/BRL). cDNA was quantified using a DNA Dipstick™ Kit (Invitrogen, San Diego CA). Equal quantities of cDNA from each sample (50 ng per reaction) were amplified by PCR for 40 cycles at 60°C annealing (1 min) and 72°C elongation (2 min) using cytokine primer pairs shown in Table 1. PCR products were separated on 1.2% agarose gels and transferred to positively charged nylon membranes (Boehringer Mannheim) by Southern blotting (122).

Detection of Cytokine mRNA

Specific PCR products were identified using the Genius™ system of nonradioactive DNA labeling and detection (Boehringer Mannheim) according to the manufacturer's protocols. Briefly, internal oligonucleotide probes specific for each cytokine (Table 1) were labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate. After overnight hybridization at 65°C, the PCR products were detected colorimetrically using an anti-digoxigenin alkaline phosphatase conjugate in an enzyme-linked immunoassay. Concanavalin A (SIGMA)-stimulated mouse splenocytes

were used as a positive control for the primers and probes and G3PDH was used as a positive control for the isolation of mRNA (123). All nucleotide primers and probes were synthesized in the Interdisciplinary Center for Biotechnology Research DNA Synthesis Core Laboratory at the University of Florida (Gainesville, FL).

Densitometric Analysis

Semi-quantitative analyses of cytokine PCR products was done using densitometry and One-Dscan software. Sample calculations were standardized using G3PDH values to ensure that equal amounts of mRNA were present in each sample thus allowing for semi-quantitative comparison between sample bands (G3PDH values for 8 wk lacrimal and 10 wk submandibular samples are reported as raw data since they did not appear to be consistent with experimental samples). Lanes which did not exceed background were given a value of zero and subsequent sample values were determined using auto-background calculations.

Results

Tissue Histology

Lacrimal, submandibular, parotid and pancreatic tissues were surgically removed from groups of 5 NOD mice at 2 wk intervals from 8 through 20 weeks of age. A small piece of each tissue was stained with hematoxylin/eosin(H&E) and examined for leukocyte infiltration. As expected, leukocytic infiltration was observed in the pancreatic islets of 8 wk old mice and increased in severity over time. By 18 wks, few islets remained due to

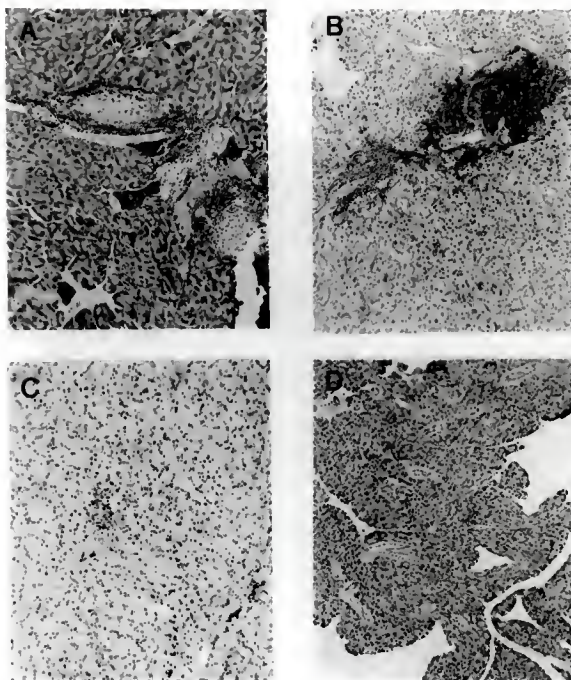


Figure 1 Histological profile of tissues showing lymphocytic infiltrates of the exocrine tissues and insulinitis in the NOD mouse. Tissue sections were stained with hematoxylin/eosin. (A) 10 wk pancreas; (B) 12 wk submandibular gland; (C) 12 wk lacrimal gland; (D) 16 wk parotid gland.

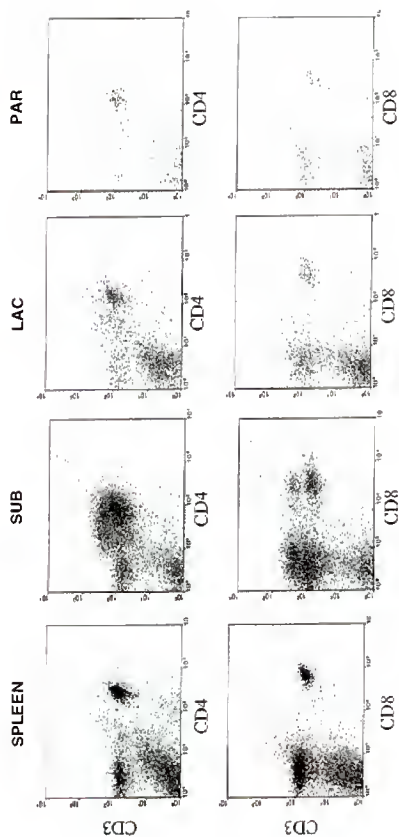


Figure 2 Flow cytometric analysis of CD4⁺ and CD8⁺ T-cell populations in lymphocytic infiltrates in tissues from 12 wk NOD mice. Spleen (SPL): CD3 - 44%, CD4 - 27%, CD8 - 11%; Submandibular gland (SMG): CD3 - 62%, CD4 - 11%, CD8 - 11%; Lacrimal gland (LAC): CD3 - 47%, CD4 - 26%, CD8 - 10%; Parotid gland (PAR): CD3 - 31%, CD4 - 13%, CD8 - 3%. Results are expressed as percentages of the total infiltrating populations.

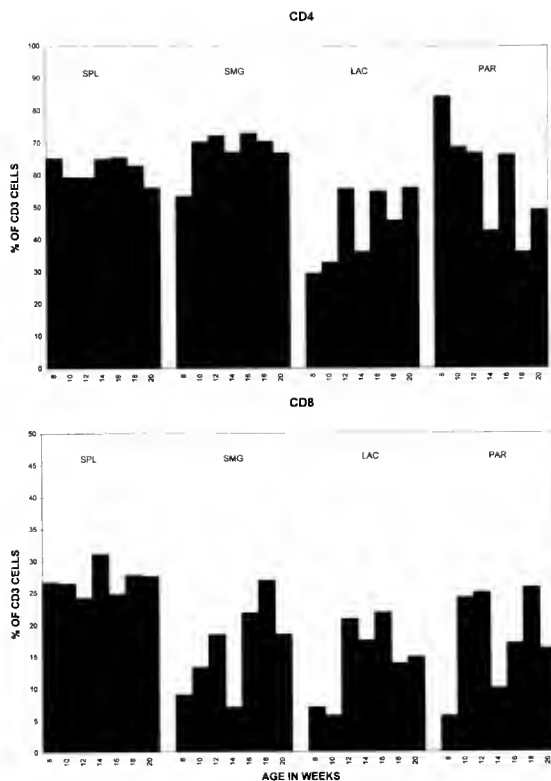


Figure 3 Histogram of the temporal expression of CD4⁺ and CD8⁺ T-cells in lymphocytic infiltrates of submandibular (SMG), lacrimal (LAC), parotid (PAR) glands and spleen (SPL) from NOD mice. Data was obtained from flow cytometric analyses of infiltrating cells using FITC-conjugated CD3 and PE-conjugated CD4 or CD8. All values are expressed as a percent of CD3⁺ cells.

autoimmune destruction. At 8 wks of age the submandibular glands of 2 of the 5 mice showed small focal areas of infiltration while the lacrimal and parotid glands remained normal. By 12 wks, the lacrimal glands showed infiltration, but their glands had fewer and smaller lymphocytic foci than the submandibular glands which were heavily infiltrated in all mice by 14 wks. In contrast, the parotid glands showed no focal lymphocytic infiltration up to 20 wks of age, although a few mononuclear cells were occasionally seen in some mice from 14 to 20 wks of age. Representative histological profiles of glandular infiltrates are shown in Fig 1.

Flow cytometric analysis of glandular infiltrating lymphocytes

Monoclonal antibodies to cell surface molecules have been widely used as phenotypic markers corresponding to functionally distinct subsets of lymphocyte populations. We have used flow cytometric analysis to determine the cellular phenotypes of lymphocytes infiltrating the pancreas, lacrimal, parotid and submandibular glands of the NOD mouse from the first appearance of lymphocytes at 8 wks through 20 wks, which is 4 wk beyond the detection of exocrine gland dysfunction. Gates set on the NOD spleen lymphocyte populations were used to select the infiltrating populations of the other tissue samples.

The percentage of the cell populations falling in this gated window remained fairly constant throughout the time course of the study averaging approximately 73% for spleen, 47% for islets, 59% for submandibular gland, 24% for lacrimal gland and 17% for parotid gland. Within these gated populations the percentage of CD3⁺ cells also remained relatively constant over the time course of the study at 48% for spleens, 41% for islets,

37% for lacrimal glands and 39% for parotid glands. However, in the submandibular gland, there was an increase in CD3⁺ cells from 41% at 8 wks to 70% at 20 wks.

Double labeling of the infiltrating populations with CD3/CD4 or CD3/CD8 is shown in Fig 2 for the 12 wk time point. Similar profiles were obtained for each time period to determine the percent of CD4 or CD8 cells (Fig 3). The CD4⁺ population in the submandibular gland approximated that in the spleen: 68% and 62% respectively. In the lacrimal gland, the CD4⁺ population was about half that of the spleen at 8 wks (~30%) and increased over time to the level of the spleen. The parotid gland showed a decrease from 84% CD4⁺ cells at 8 wks to ~50% at 20 wks. However, the low total number of lymphocytes detected in the parotid glands as well as their similarity to splenic profiles may be indicative of contamination by lymphatic vessels which are interspersed throughout the gland. There was greater variability in the infiltrating CD8⁺ populations of the exocrine glands than seen in CD4⁺ cells (Fig 3). Generally, fewer than 10% of the CD3⁺ population were CD8⁺ in these glands at 8 wks -- considerably lower than the 27% found in the spleen. As the disease progressed, the percentage of CD8⁺ cells increased in all 3 glands, but it is unclear if the apparent decrease at 20 wks is significant.

As indicated by the spleen population, the ratio of peripheral CD4⁺ to CD8⁺ cells remained relatively constant at ~2.5:1. In the submandibular gland, the CD4:CD8 ratio was 6:1 at 8 wks, and decreased to an average of 3.5:1 from 12 wks on. Both the lacrimal and parotid glands also had higher ratios in early infiltrates, but decreased to spleen levels at 12 wks.

The CD4⁺ and CD8⁺ populations did not account for all of the CD3⁺ cells. The CD3⁺, CD4⁻CD8⁻ population accounted for 5% of the spleen cells, 8% of the

Table 2. Percentage of CD3 and B220 cells in the lymphocytic infiltrates of submandibular (SMG) and lacrimal (LAC) glands of NOD mice.

TISSUE	ANTIBODY	AGE IN WEEKS							
		8 ^{a)}	10 ^{a)}	12 ^{b)}	14 ^{a)}	16 ^{b)}	18 ^{b)}	20 ^{b)}	
SMG	CD3	31.7	49.3	59.1	54.0	60.7	63.5	60.6	
	CD3+B220+	0.5	0.6	3.4	0.6	8.4	3.9	6.9	
	CD3+B220-	31.2	48.7	55.7	53.5	52.2	59.6	53.8	
	CD3-B220+	1.3	14.6	13.9	12.3	15.7	23.1	16.5	
LAC	CD3	25.1	36.1	39.5	28.7	24.6	28.5	32.7	
	CD3+B220+	0.4	0.4	2.7	0.6	3.7	4.3	2.6	
	CD3+B220-	24.7	35.7	36.8	28.2	21.0	9.3	13.4	
	CD3-B220+	0.9	4.4	33.5	3.5	40.9	27.5	30.7	

a) Flow cytometric data were collected using FITC-conjugated CD3 and PE-conjugated B220.

b) PE-conjugated CD3 and FITC-conjugated B220 were used.

Table 3. CD45RB expression in lymphocytic infiltrating cells of submandibular (SMG) and lacrimal (LAC) glands of NOD mice.

TISSUE	ANTIBODY	AGE IN WEEKS								
		8	10	12	14	16	18	20		
SMG ^{a)}	CD3	45.6	52.9	68.0	61.3	65.7	72.8	67.3		
	CD3+CD45RB/h+	3.1	13.5	14.7	15.0	18.9	23.4	17.2		
	CD3+CD45RB/l+	14.6	18.7	27.5	26.0	24.7	30.4	27.8		
	CD3-CD45RB/h+	16.1	28.4	24.7	25.8	26.0	22.6	23.6		
LAC ^{a)}	CD3	29.6	43.2	47.9	38.1	30.3	30.4	43.8		
	CD3+CD45RB/h+	1.5	7.2	12.0	5.6	10.0	10.6	7.2		
	CD3+CD45RB/l+	9.9	10.0	10.0	21.3	8.6	21.6	19.4		
	CD3-CD45RB/h+	6.7	18.8	41.6	17.1	50.2	12.2	18.7		

a) Flow cytometric data were collected using FITC-conjugated CD3 and PE-conjugated CD45RB.

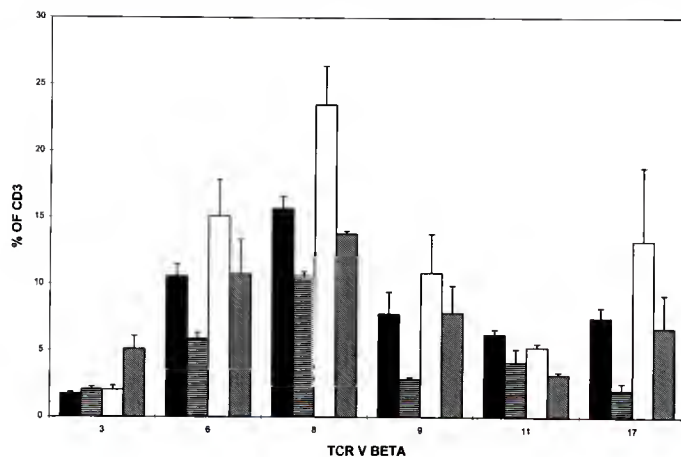


Figure 4 Histogram of selective TCR V β distribution in infiltrating lymphocytes isolated from spleen, pancreatic islet, submandibular, and lacrimal glands of NOD mice. Data from flow cytometric analyses of 8 through 20 wk samples were averaged for each tissue. Spleen-solid bar; Pancreatic islet-horizontal striped bar, Submandibular gland-clear bar; and Lacrimal gland-diagonal striped bar. Data are presented as percentage of CD3⁺ cells $\pm$ SE of the mean.

submandibular cells, 15% of the lacrimal cells and 9% of the parotid cells. This indicates an increase in the double negative population in the exocrine tissues over that of the periphery.

The infiltrating populations of the submandibular and lacrimal glands were further characterized using antibodies against the B-cell marker B220 (63) and the activation/memory marker CD45RB (123). As shown in Table 2, the CD3⁺B220⁺ B-cell population in the submandibular gland increased from ~1% at 8 wks to ~15% at 10 wks and maintained that level throughout the rest of the study. In the lacrimal gland, CD3⁺B220⁺ cells also started at 1% at 8 wks and increased to ~33% at 12 weeks. Whether the fluctuation seen between 10 and 14 weeks is meaningful is unknown at this time. In addition, the presence of a CD3⁺B220⁺ cell population was detected in both of these tissues. This phenotype did not appear in significant numbers until 16 wks of age in either gland and never exceeded 10% in the submandibular gland or 5% in the lacrimal gland. The CD3⁺B220⁺ phenotype has been reported as a double negative population in MRL/lpr mice (63) and as a lymphokine activated killer phenotype (LAK cell) in other studies (124).

CD45RB staining presented a much more complex picture (Table 3). In submandibular glands at 8 wks, ~3% of the CD3⁺ cells were CD45RB^{hi} (naive T-cell) and from 10 wk on, this CD45RB^{hi} population remained relatively stable at ~16%. The percentage of CD3⁺CD45RB^{lo} (memory T-cells) population was 15% at 8 wks, showed a 1.8-fold increase to 27% at 12 wks and remained at that level through 20 wks. The CD3⁺CD45RB^{hi} population in the submandibular gland remained constant at ~25% from 10 - 20 wks. In the lacrimal gland, the picture was less clear due to a smaller sample size

(Table 3). In general, the CD45RB^{hi} population (both CD3⁺ and CD3⁻) increased to a maximum of ~55% at 12 wks, plateaued and then decreased at 18 and 20 wks to ~24%. The CD3⁺CD45RB^{lo} population remained fairly constant at ~20% from 10 wks on.

Analysis of TCR V β usage was done using the monoclonal antibodies listed in the Materials section. It has been previously reported that the majority of the infiltrating populations of the submandibular and lacrimal glands are TCR $\alpha\beta$ (64). In the V β s tested, there was no appreciable time-related variation in response, therefore, the data from all time points were averaged for V β distribution (Fig 4). The submandibular and lacrimal glands showed a similar distribution pattern to that seen in the spleen. Both V β 6 and V β 8 were significantly increased over background as represented by V β 3, while, to a lesser extent, V β 9 and V β 17 were also increased. These increases were much more dramatic in the submandibular gland than in the lacrimal gland which paralleled the response in the periphery as represented by the spleen.

Cytokine mRNA expression in salivary and lacrimal tissues

Temporal expression of mRNA transcripts for selected cytokines expressed within the infiltrates of lacrimal and submandibular tissues from each experimental age group was determined through the use of semi-quantitative RT-PCR. PCR bands were quantified using densitometry and compared against PCR bands of G3PDH to provide a measurement of temporal changes in the production of mRNA transcripts and provide a detailed analysis of cytokine expression throughout the progression of the autoimmune activity. As presented in Fig 5, mRNA transcripts for a number of interleukins were detected generally at both an earlier age and with greater intensity in the lacrimal glands

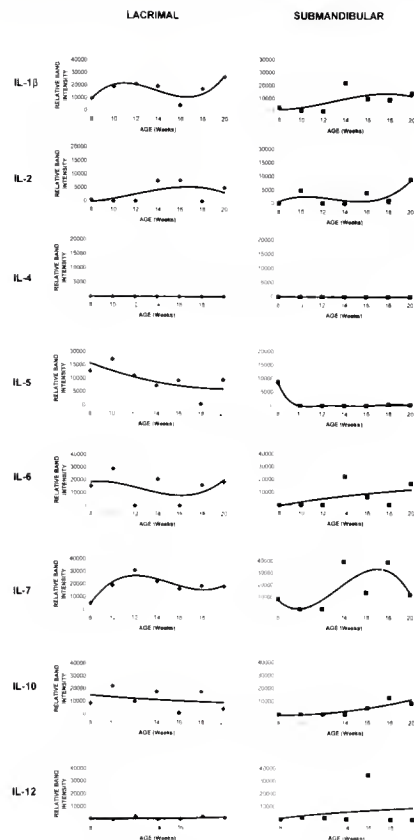


Figure 5 Interleukin mRNA expression of lacrimal and submandibular glands as determined by RT-PCR and Southern blotting. Blots were scanned and analyzed by densitometric comparison using G3PDH as a standard. Values were plotted by arbitrary density units and analyzed using a best-fit polynomial or exponential trendline.

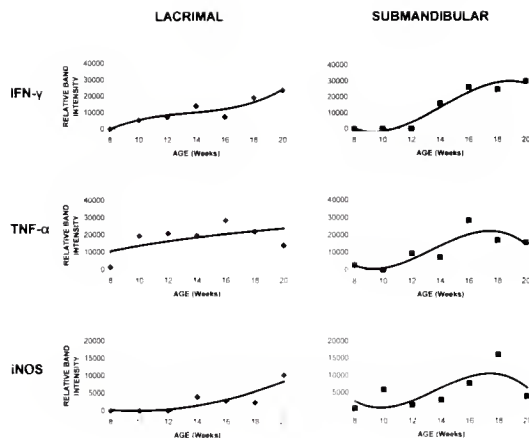


Figure 6 Proinflammatory mRNA expression of lacrimal and submandibular glands as determined by RT-PCR and Southern blotting. Blots were scanned and analyzed by densitometric comparison using G3PDH as a standard. Values were plotted by arbitrary density units and analyzed using a best-fit polynomial or exponential trendline.

than submandibular glands. In the lacrimal tissue, IL-1 β , IL-5, IL-6, IL-7, and IL-10 mRNA transcripts were detected as early as the 8 wk as well as at most later time points. In the submandibular gland infiltrates, detectable interleukin mRNA transcripts were usually not observed before 14 wk of age. Of note, mRNA transcripts for IL-4 were absent from lacrimal glands, while IL-4 and IL-5 were absent from the submandibular glands. Both interleukin transcripts were detected in control mRNA obtained from ConA stimulated NOD splenocytes, while IL-4 is commonly seen in islet infiltrates (122). mRNA transcripts for three effector cytokines, IFN γ , TNF α and iNOS were detected in both lacrimal and submandibular gland infiltrates of mice aged 8 and 12 wks, respectively (Fig 6). All three cytokines exhibited increased levels of mRNA transcription through 16-18 wks. Cytokine mRNA transcripts were rarely detected in control parotid tissues. Considered *in toto*, these results indicate that major temporal changes in the cytokine profiles occur in the lacrimal and submandibular glands of NOD mice between 12 and 16 wks of age. In the case of the submandibular glands, this is several weeks following the first appearance of lymphocytic infiltration.

Discussion

Overt diabetes in our NOD mouse colony generally begins at 12 wks of age, while the first appearance of focal lymphocytic lesions in the submandibular and lacrimal glands begins at 8-10 wks and 10-12 wks of age, respectively. Despite the early appearance of lymphocytic infiltrates within the submandibular and lacrimal glands, loss of saliva flow and tear production does not occur until 14-16 wks of age (71). For this reason, the

argument has been advanced that the autoimmune attack against the salivary and lacrimal tissues is merely secondary to the loss of immunological tolerance for a pancreatic β cell antigen also expressed on the salivary and lacrimal glands. A second argument suggests that the decreased saliva and tear flow is a consequence of the loss of blood glucose regulation.

Several recent studies have raised serious questions with both arguments. First, Leiter et al. (101) have reported that development of immunological tolerance against the β cell following intrathymic injection of islet cell homogenates into neonatal NOD mice prevented the development of diabetes, but not the autoimmune attack against the salivary glands. Second, Garchon et al (81) have linked sialoadenitis, hyper-IgG production and peri-insulinitis to a telomeric locus, but insulinitis and diabetes to a centromeric locus on chromosome 1. Third, I have observed (unpublished data) that loss of secretory function in the salivary glands precedes the onset of changes in blood glucose levels and diabetes in NOR/chr 9 mice, a strain which has delayed onset diabetes (85). These studies indicate diabetes and salivary/lacrimal gland complications in NOD mice are most likely distinct entities.

In the present study, I have attempted to define the temporal development of the autoimmune attack against the salivary and lacrimal glands of NOD mice. A unique feature of this pathogenesis, and comparable to Sjögren's Syndrome in humans, is the loss of secretory function resulting in clinical presentations of xerostomia and xerophthalmia (71,11). As such, I believe these data will allow for a comparison of the autoimmune processes, e.g., lymphocyte phenotypes, activation states and cytokine profiles, within

several organs of the same host as well as a comparison between this animal model and Sjögren's Syndrome.

Analyses of the lymphocytic infiltrates of the salivary and lacrimal glands uncovered a relatively high percentage of CD4⁺ T-cells, as might be expected from previous studies on the islet infiltrating lymphocyte populations (68). In contrast, only 10% of the T-cells detected were CD8⁺, and even fewer were CD3⁺/CD4⁺CD8⁻ double-negative T-cells. While generally accepted that CD4⁺ T-lymphocytes are necessary for the immunopathogenesis of diabetes in the NOD mouse, the role of the CD8⁺ population, particularly in the initiation of the disease, has remained more elusive (92). T-cell transfer studies in NOD mice have confirmed the necessity of CD4⁺ T-cell populations to transfer autoimmunity, whereas neither purified CD8⁺ T nor B-cells were capable (85).

It is becoming increasingly apparent that the activation state of transferred lymphocyte populations is critical. Using the CD45RB as a marker of lymphocyte activation, I observed that the majority of T-cells present in the infiltrates of both submandibular and lacrimal glands were either of a memory (CD45RB^{lo}) phenotype or a phenotype (CD45RB^{hi}) suggesting transition to an activated stage. The presence of a distinct CD45RB^{hi} population throughout the time course of this study suggests that a population of naive T-cells are being actively recruited to the salivary and lacrimal glands.

Interestingly, a recent study has shown that the ability to transfer diabetes in NOD mice is associated with a CD45RB^{lo} memory T-cell population and a concomitant increase in the IFN γ to IL-4 ratio (87). T-cells that produce IL-4 have consistently failed to transfer disease. Markedly increased levels of IFN γ mRNA transcripts were observed in both the

submandibular and lacrimal glands, possibly indicating a switch in the cytokine production by activated CD45RB^{lo} T-cells.

In addition to exploring the lymphocytic activation states, I have also investigated selected V β TCR phenotypes of the infiltrating cells. While most V β TCR populations were detected in the salivary and lacrimal gland infiltrates (as expected in the NOD mouse), an increased percentage of V β 6 and V β 8 expression was present, consistent with previous observations of preferential TCR usage observed immunohistochemically (89). The importance of V β 8 and V β 6 lymphocytes in the development of autoimmunity in both NOD and MRL/lpr mice has been demonstrated through the use of both T-cell transfer and specific anti-V β therapy (64,65,125).

Differences in the numbers of B lymphocytes infiltrating the salivary and lacrimal glands were also observed: approximately 15% in the submandibular gland, but 33% in the lacrimal tissue. Whether the increased percentage of B lymphocytes within the lacrimal glands is due to increased B-cell proliferation or active recruitment remains unknown. However, detection of CD23 (blast-2 antigen) in the lacrimal infiltrates of Sjögren's Syndrome patients support the possibility of lymphoproliferation (34).

In this study, I have also documented several age-dependent increases in cytokine mRNA expression in the salivary and lacrimal glands. Of particular interest is the increased expression of IFN γ , TNF α , and iNOS in both salivary and lacrimal tissues starting at 12-14 wks of age and increasing to maximally detected levels by 20 wks of age. By 16 wks, high levels of IL-10 mRNA production was detected as well. The dramatic rise of each of these transcripts coincides with the time of the first evidence of the loss of saliva flow as reported in previous studies (67). Whether these cytokines are involved in

effecting loss of exocrine function directly via a cytotoxic pathway or their presence is necessary to initiate lymphocyte-mediated cell killing is not yet known.

Many recent studies have focused on the potential roles of cytokines as cytotoxic effectors of exocrine tissue destruction. Evidence indicates that IL-1, IFN γ , TNF α and nitric oxide may be key mediators in the pathogenic process of islet cell destruction in both humans and NOD mice (126-129). Of particular interest, production of high levels of IFN γ by islet infiltrating cells has been repeatedly demonstrated in NOD mice and appears tightly linked to β cell destruction (127,130). Recent studies by Mathis *et al.* demonstrated that an islet-specific Th1 T-cell clone producing high levels of IFN γ is able to rapidly transfer diabetes to young NOD recipients (88). However, when shifted in vitro to an IL-4 producing phenotype, the same T-cell clone was unable to transfer diabetes. Furthermore, IFN γ has been noted to induce cell death of both islet and salivary gland cells in vitro, and may represent a non-specific mediator of exocrine cell destruction in vivo (45). However, a caveat to this argument is the detection of high levels of IFN γ and TNF α in the salivary glands of MRL/lpr mice which do not lose secretory function (66,54).

Unexpectedly, cytokine mRNA expression in the lacrimal glands typically appeared at an earlier age and in larger quantities than in the submandibular gland. This occurred despite the fact that both fewer and smaller focal lymphocytic lesions were present in the lacrimal glands in the earlier age groups, thus suggesting that lymphocytes infiltrating the lacrimal tissue are activated at an earlier stage than those in the submandibular gland. Alternatively, a lag time between the detection of focal lymphocytic lesions in the submandibular gland (8-10 wks) and the detection of increased cytokine mRNA

expression (12-16 wks) may indicate that the submandibular gland-infiltrates remain functionally quiescent and require a signal of activation.

In conclusion, this data demonstrate numerous similarities in both lymphocyte phenotypes (CD4/CD8 ratios, V β TCR restriction, and B220 populations) and cytokine expression (increased IL-1 β , IL-2, IL-10, TNF α and IFN γ) between the NOD mouse and other animal models for autoimmune sialoadenitis as well as Sjögren's Syndrome. Although the initiating agent for Sjögren's Syndrome remains unknown, it is often believed that extrinsic factors, i.e., viral agents, may be responsible for the breakdown of tolerance in immunologically susceptible individuals. In NOD mice, however, the necessary intrinsic elements for the breakdown of salivary gland tolerance exist in their genetic background. Using the NOD-*scid* mice, I have recently described multiple salivary gland abnormalities of NOD-*scid* mice that do not appear to be immunologically related (121). These alterations are detectable starting at 8-10 wks of age and include morphological abnormalities, aberrant gene expression, and increased proteolytic activity. While any number of immunological changes can result in aggregation of lymphocytes in exocrine tissues, such as the *lpr/ gld* mutation of MRL mice, graft vs. host models (131), TGF- β knockouts (61), and *bcl-2* overexpression (18), only with the appearance of developmental defects or extrinsic destruction of the exocrine tissue leads to the development of secretory dysfunction through loss of immune regulation.

CHAPTER 3
GENETICALLY PROGRAMMED DEVELOPMENT OF SALIVARY GLAND
ABNORMALITIES IN THE NOD (NON-OBESE DIABETIC)-*SCID* MOUSE IN THE
ABSENCE OF DETECTABLE LYMPHOCYTIC INFILTRATION: A POTENTIAL
TRIGGER FOR SIALOADENITIS OF NOD MICE

Introduction

The recent development of the NOD-*scid* congenic strain provides a unique model to investigate the role of the immune response in the pathogenesis of Sjögren's Syndrome (82). The NOD-*scid* mouse is homozygous at the *scid* (severe combined immunodeficiency) locus and thus lacks functional T and B lymphocytes. The *scid* mutation prevents the spontaneous development of sialoadenitis, insulinitis, and diabetes in these mice; however, the transfer of T lymphocytes from diabetic NOD mice to recipient NOD-*scid* mice can restore an autoimmune phenotype (85). In addition, because of its NOD background, the NOD-*scid* has impaired NK (natural killer) cell and reduced complement activity (82). Since NOD-*scid* mice share the same NOD genetic background, this model is ideal for studying the non-immune genetic factors that contribute to the development of autoimmunity. Previously, the dramatic changes in exocrine gland histology, protein synthesis, and secretory dysfunction are thought to be a direct result of the autoimmune lymphocytic component (67). By investigating exocrine gland function in the absence of functional lymphocytes, this specific aim demonstrates that temporal changes in salivary gland function occur in the absence of overt

autoimmunity. This indicates that the resulting immune response may actually be triggered by aberrant physiological changes in exocrine gland homeostasis and function.

Materials and Methods

Animals

BALB/cJ, C3H, CBA, and NOD/Uf mice were bred and maintained in the Department of Pathology's mouse facility (University of Florida, Gainesville, FL). NOD-*scid* mice were bred and housed in the Department of Pathology's transgenic mouse breeding colony. Both male and female mice ranging in age from 3 to 30 weeks were used. Maintenance of the *scid* mutation was assessed in experimental animals by flow cytometry of spleen cells and RT-PCR analysis of CD4, CD8, and TCR V β repertoire from isolated submandibular gland total RNA. NOD mice were routinely tested for blood glucose levels using Chemstrip bG reagent strips (Boehringer Mannheim, Indianapolis, IN). Consecutive elevated fasting blood glucose levels >240 mg/dl were considered onset of diabetes, after which time, the mice were maintained on daily insulin injections.

Saliva Collection and Flow Rate

Saliva was collected from control and experimental groups of male and female mice following stimulation of secretion using isoproterenol (0.20 mg/100 g body weight) and pilocarpine (0.05 mg/100 g body weight) (Sigma Chemicals, St. Louis, MO) dissolved in saline. The secretagogue cocktail was injected (0.1 ml volume) intraperitoneally and saliva was collected, starting 1 min post-injection, for 10 min from the oral cavity by

micropipette and placed into chilled 1.5 ml microcentrifuge tubes (67). Volume was determined by measurement with 200 μ l micropipettes. Saliva samples were collected from groups of 7 male or 5 female mice then frozen at -70°C until analyzed for temporal protein changes by enzyme assay, radio-receptor assay, SDS polyacrylamide gels and Western blotting.

Total Salivary Protein and α -Amylase Analysis

Saliva samples were analyzed for total protein using bovine serum albumin as the standard (132). Amylase was determined by its ability to hydrolyze starch according to published protocols (133). In brief, 500-1000 fold dilutions of saliva in phosphate-buffered saline (PBS) were added to a solution containing 0.4 g soluble starch in 60 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 0.15 M NaCl and 3 mM CaCl_2 . The reaction was stopped after 5 or 10 min by the addition of 0.045% I_2 , 0.045% KI, and 0.03 N HCl. Absorbance was measured at wavelength 620 nm. One unit of amylase was defined as the amount that hydrolyzed 1 mg starch/min/mg protein at 37°C .

EGF Analysis

Salivary epidermal growth factor (EGF) was estimated by the procedure of Booth et al. (134). Saliva was diluted 10 fold in PBS containing 0.2 mg/ml bovine serum albumin (BSA). Reactions consisting of 100 μ l diluted saliva, 100 μ l human placental microvilli membranes and 100 μ l ^{125}I -labeled human EGF were incubated for 24 hr at 4°C (67). Each reaction was then diluted with 3.5 ml ice-cold 0.1% BSA in PBS solution, centrifuged for 20 min at 7,000 g in an RC-3B Sorvall centrifuge and the quantity of

radiolabel associated with the membrane pellet determined using a Beckman gamma counter. The concentration of EGF was compared to a standard curve generated with dilutions of known quantities of human recombinant EGF. Membrane binding competition is independent of species origin for the source of EGF (135).

Isolation of Salivary Gland Tissues.

Parotid and submandibular glands were excised from mice killed by cervical dislocation. Each gland was freed of connective tissue, fat, and any lymph nodes, then homogenized in 10 mM Tris buffer (Ph 7.4) containing 100 μ M phenylmethylsulfonyl fluoride, 1 μ M leupeptin, and 100 μ M benzamidine. The slurry was then centrifuged at 100,000 g for 30 min to recover total membrane (67). The supernatant was saved and frozen until analysis for α -amylase activity.

Polyacrylamide gel electrophoresis and Western blot analysis

Total saliva proteins (5 μ g of total protein or 5 μ l of total saliva volume) were subjected to electrophoretic separation on a 1.5 mm thick 10% SDS-polyacrylamide gel (12% SDS for Western blots) using a modified Tris-Glycine system of Pugsley and Schnaitman (136). Western blots of gland lysates contained 20 μ g protein per lane. Gels were fixed and stained using Coomassie Brilliant Blue R-250 or transferred to Immobilon-P membranes (Millipore, Boston, MA) for 2 hr at 70v for Western blotting (137,138). The blocking buffer consisted of 3% nonfat dry milk and 3% BSA in Tris-buffered saline. Polyclonal rabbit anti-mouse parotid secretory protein (mPSP) IgG antibody (139), kindly provided by Dr. William Ball (Dept. of Anatomy, Howard

University) or polyclonal rabbit anti-rat proline rich protein (140), kindly provided by Dr. David Castle (Dept. of Anatomy and Cell Biology, University of Virginia), was incubated with each membrane for 2 hr at 25°C. Following three 10 min washes, the membranes were incubated in alkaline phosphatase conjugated goat anti-rabbit immunoglobulin (Sigma Chemical Co.) and exposed to substrate as previously described (95).

Protein Sequencing

Salivary proteins were subjected to electrophoresis on 10% SDS-polyacrylamide gels as described above and transferred to Immobilon-P membranes. Selected protein bands were carefully cut from the membranes and subjected to N-terminal sequencing using Applied Biosystems Model 470A Gas Phase Protein Sequencer with Model 120A on-line PTH analyzer (University of Florida ICBR Protein Sequencing Core Laboratory). Protein sequences for the first 12 amino acids of each protein were entered into a protein database for comparison with known protein sequences.

Histology

Freshly excised submandibular glands were placed immediately into 10% PBS-buffered formaldehyde (pH 7.2). Each tissue was embedded in paraffin, sectioned, then stained with hematoxylin/eosin dyes (UF Diagnostics Referral Laboratory, Gainesville, FL). The stained sections were viewed using light microscopy.

Statistical Analyses

All measures of variance are given as standard deviations of the mean. Tests of significance for differences between independent means were performed with the unpaired Student *t*-test. Results in which $p < 0.05$ were considered significant.

Results

Analysis of NOD-*scid* Saliva

To determine the impact on salivary function of the *scid* mutation in the NOD mouse, I analyzed whole saliva samples from individual NOD-*scid* mice for total volume, total protein, amylase activity and EGF concentration. Saliva from 10-12 wk old NOD-*scid* mice, an age at which salivary glands in NOD mice are devoid of detectable lymphocytic infiltration, was compared to that of >20 wk old NOD-*scid* mice, an age when lymphocytic infiltration is present in the salivary glands of NOD mice (Table 4). While there was no significant age or sex differences observed for salivary flow rates ($P > 0.05$), significant sex-related differences were observed for protein content ($P < 0.05$). Saliva from female NOD-*scid* mice contained approximately 40% less total protein than that of male mice in both experimental groups.

Saliva from NOD-*scid* mice were analyzed for two proteins, EGF (a product of submandibular gland ductal cells) and amylase (a product of parotid gland acinar cells), normally secreted in high quantities. As presented in Table 4, the quantity of EGF in the saliva from >20 wk old male NOD-*scid* mice showed a 21% decline compared to the amount present in 10-12 wk old male mice ($P < 0.002$). A comparison of amylase activity

Table 4
Analysis of NOD-*sicid* Saliva

Age of Mice	Total Salivary Volume (μ l/10 min)	Total Protein (mg/ml)	EGF ^a (ng/ml)	Amylase Activity (mg starch/min/ml)
10-12 weeks (n=12)	271 $\pm$ 92 ^b	1.9 $\pm$ 7.7	nd	1140 $\pm$ 188
Male (n=7)	240 $\pm$ 98	2.3 $\pm$ 8.1	563 $\pm$ 38	1099 $\pm$ 156
Female (n=5)	314 $\pm$ 79 ^{d,♦♦}	1.8 $\pm$ 1.5 ^{d,*}	nd	1181 $\pm$ 231 ^{d,♦♦}
>20 weeks (n=12)	329 $\pm$ 58 ^{e,♦♦}	1.9 $\pm$ 6.7 ^{e,♦♦}	nd	635 $\pm$ 176 ^{e,***}
Male (n=8)	321 $\pm$ 41	2.3 $\pm$ 3.8	443 $\pm$ 45 ^{e,***}	590 $\pm$ 162
Female (n=4)	345 $\pm$ 90 ^{d,♦♦}	1.1 $\pm$ 1.4 ^{d,***}	nd	680 $\pm$ 202 ^{d,♦♦}

^an = 6 males and n = 6 females for EGF determinations.

^bValues are given as the mean $\pm$ standard deviation.

^cnd, not determined.

^dStatistical comparison of female to male values by the unpaired Student *t* test : (**P* < 0.05, ***P* < 0.002, ****P* < 0.0002, ♦♦ *P* = NS).

^eStatistical comparison of > 20 week NOD-*sicid* values to 10- to- 12 week values by the unpaired Student *t* test : (**P* < 0.05, ***P* < 0.002, ****P* < 0.0002, ♦♦ *P* = NS).

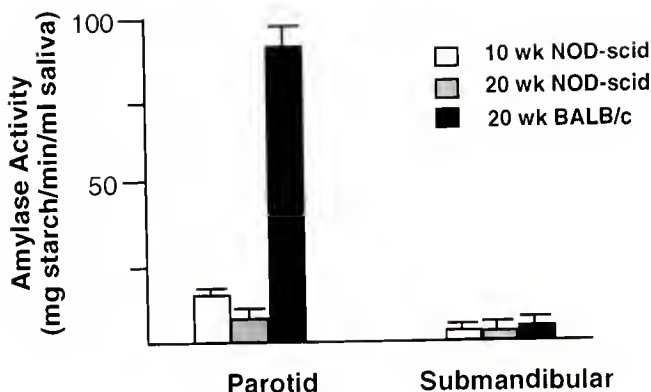


Figure 7 Cytoplasmic amylase activity of parotid and submandibular glands. Homogenates of parotid and submandibular glands were centrifuged at 100,000 x g. Lysates prepared from the glands of 10-12 wk old NOD-*scid* (open bars), >20 wk old NOD-*scid* (hatched bars), and 20 wk old BALB/cJ mice (solid bars) were tested for amylase activity in a starch hydrolysis assay. Values are expressed as means of 4 experimental animals performed in triplicate $\pm$ the standard deviations.

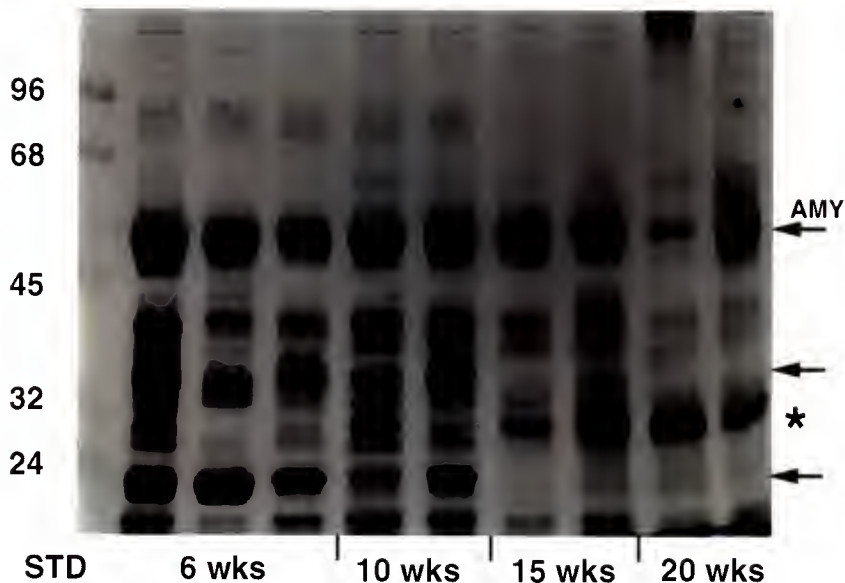


Figure 8 Temporal changes in the protein profiles of saliva from NOD-*scid* mice. Saliva was collected from 6 wk old NOD-*scid* (Lanes 1-3), 10 wk old NOD-*scid* (Lanes 4 and 5), 15 wk old NOD-*scid* (Lanes 6 and 7), and 20 wk old NOD-*scid* mice (Lanes 8 and 9) following isoproterenol/pilocarpine stimulation. Salivary protein (5 μ g protein in 35 μ l SDS-PAGE sample buffer containing β -mercaptoethanol) was loaded on each lane and separated by electrophoresis through a 10% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. Prestained molecular weight markers (STD) were: 96 kDa, Phosphorylase B; 68 kDa, bovine serum albumin; 45 kDa, ovalbumin; 32 kDa, carbonic anhydrase; 24 kDa, soybean trypsin inhibitor.

in the saliva of old versus young male and female NOD-*scid* mice (Table 4) also revealed significant decreases over time. Again, no sex-related differences in amylase activity were observed, with both male and female NOD-*scid* mice showing approximately 50% declines in activity between the two age groups ($P < 0.0002$).

Analysis of Cytoplasmic Amylase

The temporal decline in amylase activity present in saliva of NOD-*scid* mice was also reflected in the amylase activity present in parotid and submandibular gland lysates (Fig 7). Cytoplasmic fractions from parotid gland lysates showed a 50% reduction in amylase activity in the >20 wk old NOD-*scid* mice when compared to those of 10-12 wk NOD-*scid* mice and a 90% reduction when compared to levels obtained from parotid gland lysates of BALB/c mice. As expected, submandibular gland lysates exhibited low levels of amylase activity in all experimental groups.

Temporal Changes in NOD-*scid* Salivary Proteins by SDS-PAGE

To evaluate temporal changes of salivary proteins in NOD-*scid* mice, we compared electrophoretic separations in SDS-polyacrylamide gels of three individual saliva samples collected from 6 wk old female NOD-*scid* mice, plus two samples collected from each of 10 wk, 15 wk, and 20 wk old female animals (Fig 8). Although the total protein concentration in each saliva sample is constant, the gel profiles revealed substantial differences in protein composition between animals of the various age groups. These protein profiles were similar whether constant protein (5 μ g) or constant volumes (5 μ l) were applied to the SDS-PAGE. The temporal decline (then precipitous disappearance

from the saliva samples of 20 wk old NOD-*scid* mice) of the protein observed at approximately 55 kDa, shown to be amylase (105), is consistent with reported changes observed for salivary amylase in NOD mice when analyzed on SDS-PAGE gels.

Two additional protein bands, one at 32 kDa and one at 20 kDa (indicated by arrows in Fig 8), present in relatively high amounts in saliva of NOD-*scid* mice of 10 wks of age or less, virtually disappear from saliva of NOD-*scid* mice 15 wks of age and older. Interestingly, the decline of these two proteins appeared to correlate with the emergence of a third protein band at approximately 27 kDa (indicated by an asterisk in Fig 8). By 20 wks of age, this 27 kDa protein band appears to be one of the most abundant proteins in the salivary samples.

Sequence Homology of the 20 kDa, 27 kDa and 32 kDa Proteins with Parotid Secretory Protein

N-terminal amino acid sequence analyses of the 20 kDa, 27 kDa and 32 kDa protein bands indicated that all three were homologous to parotid secretory protein (PSP) (Fig 9). Interestingly, the N-terminal amino acid residues of both the 32 kDa and 20 kDa proteins, the two isoforms observed prior to the time NOD mice present with sialoadenitis, were not only identical to each other, but were also identical to the published murine PSP sequence beginning at the signal cleavage site of the secretory form (108;141-142). In contrast, the N-terminal sequence of the 27 kDa protein, appearing at a time NOD mice begin exhibiting sialoadenitis, matched an internal portion of the PSP starting at the 27th amino acid after the signal cleavage site. The secretory form of PSP has been

reported to be a 20 kDa, leucine-rich glycoprotein that is abundant in the secretory fluids of the parotid gland (105).

Antigenic Cross-reactivity of Salivary PSP from NOD-*scid*, NOD and BALB/c Mice

To confirm the identity of the 20 kDa, 27 kDa and 32 kDa protein bands as PSP, Western blots of SDS-PAGE separated saliva from 10 wk old NOD-*scid*, 20 wk old NOD-*scid*, diabetic NOD and normal BALB/c mice were treated with anti-PSP polyclonal antibody. As shown in Fig 10, anti-PSP antibody detected each of the three isoforms. Saliva from BALB/c and 10 wk old NOD-*scid* mice contained predominately the 20 and 32 kDa isoforms, while saliva from diabetic NOD contained the 27 and 20 kDa isoforms and lacked the 32 kDa isoform. Saliva from 20 wk old NOD-*scid* mice contained each of the isoforms, but had greatly reduced levels of both the 20 and 32 kDa isoforms compared to the BALB/c and 10 wk NOD-*scid* mice.

PSP Detected in the Cytoplasmic Fractions of Salivary Glands of NOD-*scid* Mice

Cytoplasmic lysates of the submandibular and parotid glands were analyzed on Western blots for the presence of PSP using anti-PSP antibody (Fig 11). Lysates of submandibular glands from 10 wk old NOD-*scid* mice revealed the presence of all three PSP isoforms, and each isoform increased in quantity by 20 wks of age (Lane 2 versus Lane 3). No PSP was present in the lysates of BALB/c submandibular glands (Lane 1).

Lysates of parotid glands from 10 wk old NOD-*scid* mice contained the 32 kDa protein band plus a comparatively low level of the 20 kDa protein (lane 5). In contrast, by 26 wks of age, parotid gland lysates from NOD-*scid* mice exhibited all three isoforms

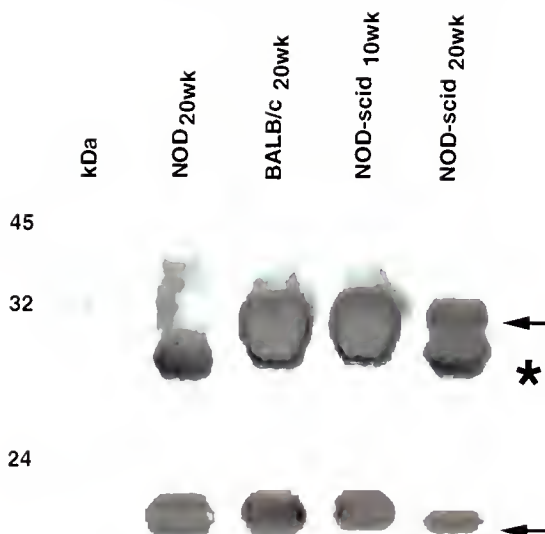


Figure 10 Identification of parotid secretory protein in saliva using polyclonal anti-PSP antibody on Western blots. Protein (5 μ g/lane) in whole saliva from 20 wk old diabetic NOD (Lane 1), 20 wk old BALB/c (Lane 2), 10 wk old NOD-*scid* (Lane 3, and 20 wk old NOD-*scid* mice (Lane 4) was separated using SDS-PAGE, electroblotted to Immobilon-P membranes, and reacted with rabbit anti-mouse PSP antibody. The Western blots were then developed using alkaline phosphatase-conjugated anti-rabbit IgG plus substrate. The 32 kDa and 20 kDa bands are indicated with arrows and the 27 kDa protein with a star. Prestained molecular weight markers are 45 kDa, ovalbumin; 32 kDa, carbonic anhydrase; and 24 kDa, soybean trypsin inhibitor (Lane S).

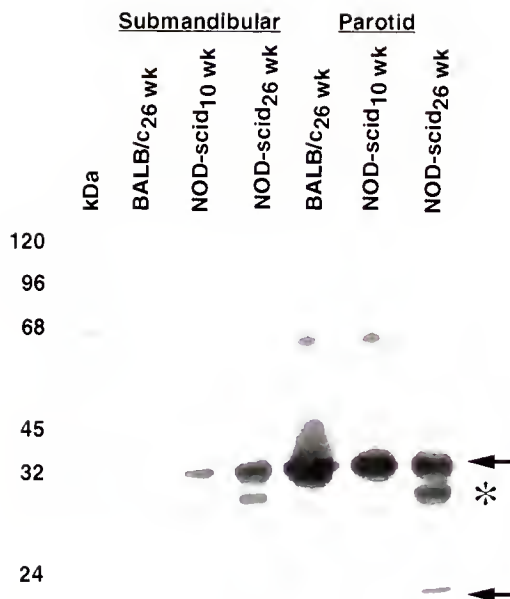


Figure 11 Representative Western blot analysis of cytoplasmic fractions of submandibular and parotid cell lysates using anti-PSP polyclonal antibody. The cytoplasmic proteins (20 μ g/lane) of either submandibular gland lysates prepared from 26 wk old BALB/c (Lane 1), 10 wk old NOD-*scid* (Lane 2), and 26 wk old NOD-*scid* mice (Lane 3) or parotid gland lysates prepared from 26 wk old BALB/c (Lane 4), 10 wk old NOD-*scid* (Lane 5), and 26 wk old NOD-*scid* mice (Lane 6) were separated on a 12% polyacrylamide gel and transferred to Immobilon P membranes. Membranes were treated with alkaline phosphatase-conjugated rabbit anti-mouse PSP and substrate. The 32 kDa and 20 kDa protein bands are indicated with arrows, while the 27 kDa protein band is shown with a star. Prestained molecular weight markers are the same as for Fig 9.

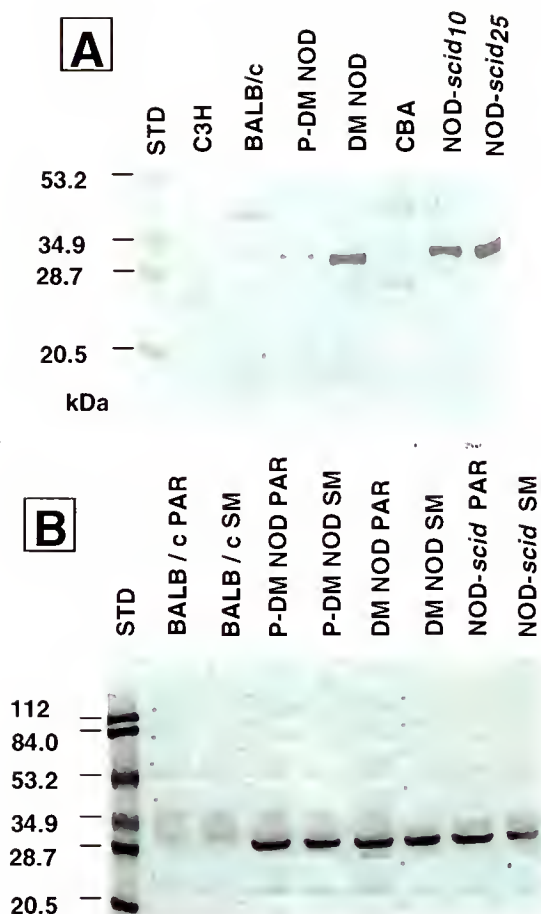


Figure 12 Western blot analysis of saliva and cytoplasmic fractions of parotid and submandibular cell lysates using anti-Proline-Rich Protein polyclonal antibody. Panel A, 10% SDS-PAGE of constant saliva protein of 5 µg from mouse strains C3H, BALB/c, CBA, NOD, and NOD-*scid*. P-DM, prediabetic NOD; DM, diabetic NOD. Panel B, represents the antibody staining of PRP in parotid (PAR) and submandibular (SM) gland lysates from BALB/c, P-DM and DM NOD, and NOD-*scid* mice. Each lane contained 20 µg of protein from total cell lysate.

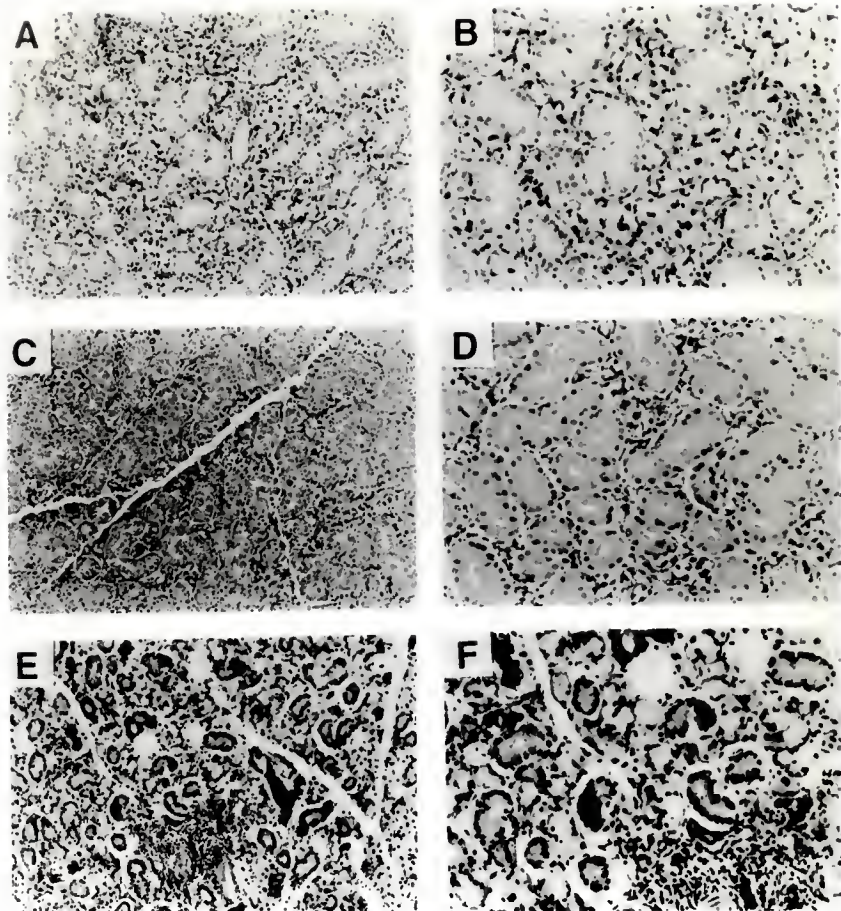


Figure 13 Morphological changes in the salivary glands of NOD-*scid* mice. Hematoxylin/eosin-stained tissue sections of submandibular glands from 10 wk old NOD-*scid* (A: 100X, B: 200X), 30 wk old NOD-*scid* (C: 100X, D: 200X) and 20 wk old NOD mice (E: 100X, F: 200X).

(lane 6). Again, lysates of BALB/c parotid glands contained only the 32 kDa protein (lane 4), comparable with the profile of young NOD-*scid* mice. Interestingly, the anti-PSP antibody bound to a protein band of approximately 65 kDa present in the parotid gland lysates of both BALB/c and 10 wk old NOD-*scid* mice; however, this protein band has not been studied. These results show the temporal nature of the appearance of the 27 kDa isoform of PSP in the salivary glands and the abnormal expression of PSP by submandibular glands of NOD-*scid* mice.

Detection of Proline-Rich Protein (PRP) in the Saliva and Salivary Glands of NOD and NOD-*scid* mice

The PRPs are a set of proteins whose synthesis are induced in the salivary glands of mice following chronic β -adrenergic agonist treatment or by the introduction of deleterious dietary changes (104). Saliva from NOD-*scid*, diabetic or prediabetic NOD mice, as well as BALB/c, CBA, and C3H strains were evaluated by Western blot for the presence of PRP. As indicated in Fig 12A, antibody to rat PRP was able to cross-react with a protein of approximately 33 kDa in saliva of diabetic NOD, 10 wk and 25 wk NOD-*scid* mice. In contrast, very little of this protein was detectable in the saliva from control BALB/c and prediabetic NOD mice. No reactivity was detected in the saliva of C3H or CBA mice. N-terminal amino acid sequence analysis confirmed the identity of this protein as PRP (data not shown). An examination of parotid and submandibular gland lysates revealed that PRP expression was occurring in both glands of the NOD-*scid*, prediabetic, and diabetic NOD mice, but not in control BALB/c animals (Fig 12B).

Histological Changes in the Submandibular Glands of NOD-*scid* mice

Histological sections of submandibular glands from 15 individual NOD-*scid* mice ranging in age from 3 to 30 wks were evaluated for structural changes. Figure 13 shows hematoxylin/eosin stained sections of submandibular glands from 10 wk (Fig 13A & 13B) and 30 wk (Fig 13C & 13D) old female NOD-*scid* mice. A comparison of these two time points reveal a marked, progressive loss of acinar tissue and a decline in the acinar to ductal cell ratio. The histology of the parotid gland was also examined. However, there were no observable changes in the cellular or glandular morphology between 10 wk and 30 wk NOD-*scid* mice (data not shown) as was seen in the submandibular gland. As expected, the NOD-*scid* tissue displayed no signs of immune cell infiltration. In contrast, submandibular gland tissue from diabetic NOD mice, which appear to have only slight loss of acinar tissue, is highly infiltrated with mononuclear cells (Fig 13E & 13F).

Discussion

The clinical symptom of xerostomia, or oral dryness, associated with human Sjögren's Syndrome is commonly attributed to two problems: the loss of the fluid phase of saliva and changes in salivary protein composition (1). Both problems are considered a result of a progressive autoimmune response against the salivary glands. This hypothesis is supported by studies using the NOD mouse model in which changes in both salivary flow rates and protein composition are associated with the appearance of immune cells in the salivary glands (67). Unfortunately, these studies in man and the NOD mouse fail to

determine if the loss of salivary function is a direct result of an autoimmune attack or if the autoimmunity is in response to specific changes in the exocrine glands.

In the present study, I have attempted to dissociate immune and non-immune factors which may contribute to the loss of salivary gland function by using the NOD-*scid* mouse. As expected, histological analyses of salivary glands from NOD-*scid* mice did not reveal the presence of lymphocytic infiltrates. Furthermore, total salivary flow and protein concentration measured in NOD-*scid* mice appeared similar to the published values of BALB/c mice and prediabetic NOD mice (67). The composition of saliva proteins, as shown by EGF and amylase concentration in NOD-*scid* saliva, however, showed significant changes with age, whereas the levels detected in BALB/c saliva remained relatively constant over the same time period (67,71). This was further reflected by greatly reduced amylase activity (90% reduction) in parotid gland lysates of NOD-*scid* mice as compared to BALB/c. In addition, amylase activity detected in NOD-*scid* parotid lysates declined nearly 50% between 10 and 25 wks of age. Temporal analyses of NOD-*scid* saliva by SDS-PAGE again revealed the age related decline of amylase as well as several additional proteins. Age-related changes in murine saliva volume and composition in non-NOD strains does not typically occur until after 12 months of age (54).

The most striking findings, however, were the discovery of a novel expression of PRP and an internally cleaved PSP isoform (27 kDa) which was prominent in 15 wk NOD-*scid* saliva but was not detected in saliva from BALB/c mice or younger NOD-*scid*. The abundant appearance of these proteins may explain why total saliva protein concentration remained constant despite the decline in other major proteins. Proline-rich

protein is a latent constituent of murine saliva which is induced in response to glandular trauma (104). In normal mice, PSP is a 20 kDa, leucine-rich glycoprotein of unknown function that is secreted predominately by amylase producing acinar cells of the parotid gland (105). Studies have shown a developmental coordination of murine PSP and salivary amylase expression in the parotid gland of adult mice where the two proteins appear in constant ratios (107,109). With the onset of diabetes in NOD mice (>14 wks) and the aging of NOD-*scid* mice (>15 wks), both the 32 kDa and 20 kDa isoforms of PSP are replaced with a 27 kDa protein band detected with the anti-PSP antibody. Sequence analysis of the N-terminal amino acid residues of the 27 kDa isoform revealed that the protein started at an internal region (+27 aa) of the PSP protein. Interestingly, all three isoforms of PSP appeared in the lysates of the submandibular glands of older NOD-*scid* mice but not of normal BALB/c mice. These findings suggest that, first, the glandular specificity of PSP gene expression is lost over time in NOD and NOD-*scid* mice, and second, the transition to a new isoform of PSP might involve differential splicing and/or alteration of post-translational modifications.

Taken together, these findings suggest that both submandibular and parotid gland function, as shown by alterations in gland specific PSP and PRP gene regulation and salivary EGF and amylase concentrations are altered in NOD-*scid* animals between 10 and 25 wks of age. Therefore, changes in saliva protein content in the NOD-*scid* mice are probably not due to the insufficiencies of an individual gland but are multiglandular and potentially affecting other exocrine gland function. The low level of detection of amylase activity in parotid gland lysates, ectopic expression of PRP, and the appearance of a novel

isoform of PSP in both the parotid and submandibular glands further suggest that these changes take place at the intracellular level.

Histological examination of the submandibular gland, but not the parotid gland, of aging NOD-*scid* mice revealed a remarkable decline in the acinar to ductal cell ratio. This may be due to acinar cell loss, the hyperproliferation of ductal cells, or a combination of both. Acinar cell loss in the submandibular gland may be triggered by the observed defects in protein synthesis or processing which could potentially lead to cell death. Since whole salivary flow rates in the older NOD-*scid* animals do not decline despite this apparent loss of submandibular acini, it is possible that the parotid, sublingual, and minor salivary glands in the oral mucosa have increased salivary output in order to compensate for this loss of acinar cells (143,144). An interesting alternate hypothesis to acinar cell loss is the hyperproliferation of the submandibular ductal cells, which are known to have self-renewing capacity. Hyperplasia and metaplasia of the salivary ductal epithelium is a hallmark feature seen in labial salivary biopsies of human Sjögren's patients (15). It should be noted too that abnormal proliferation of differentiated tissue is often accompanied by the loss of differentiated cell protein synthesis and function (145). This hypothesis is especially attractive in light of studies showing that the ductal cells of the NOD exocrine pancreas can undergo hyperproliferation (146,147). Therefore, it is altogether possible that similar developmental abnormalities are occurring in the submandibular gland and pancreas of NOD mice that may precede the development of sialoadenitis and insulinitis respectively, and be inherently involved in the pathogenesis of these autoimmune lesions.

The *de novo* production of the 27 kDa PSP isoform in aging NOD-*scid* mice suggests that aberrant proteolytic processing may play a role in the generation of

otherwise hidden cryptic antigens, priming the immune system for an autoimmune response. The time at which this new isoform of PSP appears in the saliva and submandibular glands of NOD-*scid* mice coincides with the time of appearance of lymphocytic infiltrates in the salivary glands of NOD mice. In addition, morphological changes in the salivary glands of NOD-*scid* mice involving loss of acinar tissue are detectable by histology in the absence of lymphocytic infiltrates. These observations suggest that changes in the salivary glands of NOD mice occur independently of lymphocytic infiltration and that development of the associated autoimmune activity may actually occur in response to these changes. This is consistent with the proposed model for the induction of human Sjögren's Syndrome (1), except that the initial "injury" to the gland is intrinsic i.e. genetically programmed in the NOD mouse rather than extrinsic i.e. a consequence of viral infection or other insult.

A newly emerging model of pathogenesis of autoimmune sialoadenitis in the NOD mouse suggests that the initial trigger may reside in a defect in salivary gland homeostasis, leading to the production of new or altered proteins. As antigenic forms of cellular and secretory proteins are released, the immune system responds by initiating the homing of immune cells to the exocrine tissue. Production of cytotoxic cytokines and direct cell killing by activated T cells may play a role in furthering glandular damage. However, the presence of a subclass of autoantibodies recognizing the β -adrenergic and muscarinic-cholinergic receptors alludes to a potential mechanism for the decline in saliva production and protein output which involves antibody-mediated impairment of neuro-glandular stimulation. Thus, the presence of autoantibodies may be responsible for secondary effects on gland function following a primary immunological disturbance generated by a

genetic defect in the salivary glands. This model is useful in explaining why first, the presence of activated lymphocytes is necessary to develop the loss of the fluid phase of saliva in the NOD mouse while NOD-*scid* salivary flow rates remain normal, and second, how an autoimmune response against relatively small portions of the total area of the submandibular gland may dramatically affect the total salivary output of the parotid, sublingual and submandibular glands combined.

In conclusion, by using the NOD-*scid* mouse model, I have begun to dissociate elements of salivary dysfunction in the NOD mouse attributable to the progression of the autoimmune disease from normal/abnormal events dictated by the genetic background of the animal. These findings are striking in that they suggest an underlying defect in salivary gland homeostasis in NOD mice which results in a histopathological and functional phenotype similar to human Sjögren's syndrome.

CHAPTER 4

EXPRESSION OF PAROTID SECRETORY PROTEIN (PSP) IN MURINE LACRIMAL GLANDS AND ITS POSSIBLE FUNCTION AS A BACTERIAL BINDING PROTEIN IN EXOCRINE SECRETIONS

Introduction

The protein and mucin-rich secretions derived from the salivary, lacrimal, and other minor exocrine tissues, i.e. labial and hardarian glands, are essential for maintaining the health and integrity of the oral and ocular surfaces (9). For the most part, both tear and saliva secretions serve similar functions and contain many of the same protein constituents, e.g., EGF, NGF, TGF- α , lactoferrin, lysozyme, and immunoglobulins (9,10). At the same time, however, saliva and/or tear specific secretory proteins, as evidenced by salivary amylase and digestive enzymes, provide for specialized physiological functions of the individual secretory fluids (9).

Chapter 3 of this dissertation documented unique changes in the synthesis of several proteins noted specifically in saliva, including *de novo* synthesis of PSP as well as decreased concentrations of amylase and EGF in NOD mice (121). Similarly, protein expression abnormalities were detected in NOD-*scid* mice. These findings suggest that changes in saliva protein constituents in the NOD strain are independent of the autoimmune destruction of the glands. The aberrant synthesis of PSP in the submandibular glands of NOD mice led us to examine the question of whether PSP may

be abnormally synthesized in other exocrine tissue of NOD mice, and whether transcriptional splicing is likely to account for the novel PSP isoform described in Aim 3. In this specific aim, I show that while PSP is synthesized in the lacrimal glands of NOD mice, it is detected in the lacrimal glands in several other laboratory mouse strains as well. As a constituent in saliva and tears, I provide evidence of a potential anti-microbial function for PSP which may explain its normal synthesis by both the salivary and lacrimal glands. In addition, NOD mice were found to have lost the normal regulation of PSP gene transcription in a number of organs/tissues. No alternatively spliced mRNA was detected, however, the NOD PSP gene contains numerous base pair changes indicative of strain specific differences between mice.

Materials and Methods

Animals

BALB/c, CBA/J, and NOD/Uf mice were bred and maintained under SPF conditions in the mouse colony of the Department of Pathology and Laboratory Medicine at the University of Florida, Gainesville, FL. C3H/HeJ and NOD-*scid* mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Both male and female mice ranging in age from 8 to 25 weeks were used. NOD mice were routinely tested for blood glucose levels using Chemstrip bG reagent strips (Boehringer Mannheim, Indianapolis, IN). Consecutive elevated fasting blood glucose levels >240 mg/dl were considered onset of diabetes, after which time the mice were maintained on daily insulin injections (67).

NOD mice of 15 weeks of age were separated into diabetic and prediabetic sample groups for protein studies.

Bacteria

Lysteria monocytogenes and *Streptococcus mutans* were gifts from Dr. F. Southwick, Department of Infectious Diseases, University of Florida, and Dr. A. Bleiweis, Department of Oral Biology, University of Florida. *Actinobacillus actinomycetemcomitans* was generously provided by Dr. P. Fives-Taylor, Department of Microbiology, University of Vermont.

Isolation of Tissues for Protein Studies

Tissues were excised from mice killed by cervical dislocation. Glands were pooled from a minimum of two age- and sex- matched mice of each strain, freed of connective tissue, fat, and any lymph nodes, then homogenized in 10 mM Tris buffer (pH 7.4). The slurry was centrifuged at 500 g for 15 min to pellet cellular debris and protein concentrations of the resulting supernatant were determined by the method of Bradford (132) using bovine serum albumin as the standard. Supernatants were frozen at -70° C for Western blot analysis.

Isolation of total RNA

Excised tissues were immediately homogenized in 2 ml of 4M Guanidinium Isothiocyanate (GITC) as described by Chirgwin *et al.* (148). Briefly, 100 µl of 10% Sarkosyl and 14.2 µl of 2-Mercaptoethanol were added to homogenates and total RNA

was isolated by centrifugation at 35,000 g over a 2 M CsCl gradient. RNA pellets were washed in 200 μ l of 80/20% ethanol/Diethyl Pyrocarbonate (DEPC) treated water and then resuspended in 200 μ l of DEPC-treated water. RNA was precipitated through the addition of 500 μ l of 100% ethanol and 8 μ l of 5M NaCl. Quantitation of total RNA was determined by spectrophotometric analysis at 260 nm wavelength.

RT-PCR and Southern Blot Detection of PSP PCR Products

Total tissue RNA (2 μ g) was pelleted by centrifugation and reverse transcribed using Superscript II Reverse Transcriptase (Gibco BRL) (122). The resulting cDNA was amplified by PCR for 40 cycles using a 94°C denaturation (1 min), 60°C primer annealing (1 min) and 72°C elongation (2 min) using 5'GCAGAGAAACAAGGATCTCG and 3' CACTGGAGAGTAGCCAGCAGG PSP-specific primers. These primers spanned a region preceding the start codon and extending beyond the translation termination site (149). PCR products were separated on 1.2% agarose gels and transferred to nylon membrane by Southern blotting. To confirm the identity of specific PCR products, hybridization of digoxigenin-labeled oligonucleotide internal probe (5' AATGCGACCGTTCTTGCC) specific for PSP cDNA was carried out using the Genius system of Boehringer-Mannheim (Indianapolis, IN) (150). Briefly, oligonucleotide probes were labeled with digoxigenin using terminal transferase. Southern blot membranes were baked at 80°C for 1 hour, blocked with pre-hybridization buffer (Genius Kit), and hybridized with labeled probes overnight at 65°C. Colorimetric detection of PSP product was assayed using an anti-digoxigenin alkaline phosphatase conjugated antibody according to manufacturer's instructions. Primers and probes specific for G3PDH were used as

positive controls for all PCR reactions. All nucleotide primers and probes were synthesized in the University of Florida's ICBR DNA Synthesis Laboratory (Gainesville, FL).

Polyacrylamide gel electrophoresis and Western blot analysis

Gland lysates (30 µg of total protein per lane) were subjected to electrophoretic separation on a 12% SDS-polyacrylamide gel using a modified Tris-Glycine system of Pugsley and Schnaitman (136). Gels were stained by using Coomassie Brilliant Blue R-250 (137) or transferred to Immobilon-P membranes (Millipore, Boston, MA) for 2 hr at 70v for Western blotting (138). The blocking buffer consisted of 3% nonfat dry milk and 3% BSA in Tris-buffered saline. Polyclonal rabbit anti-mouse parotid secretory protein (mPSP) IgG antibody, kindly provided by Dr. William Ball (Dept. of Anatomy, Howard University) (139) was incubated with each membrane for 2 hr at 25°C. Following three 10 min washes, the membranes were incubated in alkaline phosphatase conjugated goat anti-rabbit immunoglobulin (Sigma Chemical Co.) and exposed to substrate as previously described (95).

Purification and Radiolabeling of PSP

Parotid secretory protein was purified using a one-step procedure by electrophoretic separation of saliva proteins on a 3 mm 10% polyacrylamide prep gel. Four hundred µl of whole saliva from C3H/HeJ or BALB/c mice was separated using a single large well in the stacking gel. Molecular weight standards (Bio-Rad) and 0.5 cm of the sample well were cut and transferred to PVDF membrane for Western blotting.

Following detection of PSP migration, the similar region on the remaining unfixed gel was removed, macerated by an electric homogenizer in 3 ml PBS containing 0.02% NaN_3 and 0.2% SDS, and placed on an orbital shaker overnight at 4°C. The extracted protein was dialyzed against ddH₂O and lyophilized to concentrate the purified protein. Typically, 1.5-2 µg of pure PSP was recovered for 400 µl of saliva protein. Purity of PSP was determined by electrophoresis and staining of polyacrylamide gels with Coomassie Brilliant Blue R-250, autoradiography and Western blot. Pure PSP, $M_r \sim 25,000$ was radiolabeled using chloramine-T and Na^{125}I obtained through Amersham (Arlington Heights, IL). Radiolabeled PSP was purified from free [^{125}I], potentially other contaminating proteins and radiolabeled BSA in the incubation buffer by molecular sieve chromatography on Sephadex G-75 obtained from Pharmacia (Uppsala, Sweden).

PSP Binding to Bacteria

Four strains of bacteria, *L. monocytogenes*, *E. coli*, *S. mmtans*, and *A. actinomycetemcomitans* were grown in overnight cultures at 37°C. The bacteria were pelleted by centrifugation at 4°C for 5 min at 15,000 x g and washed in PBS buffer containing 0.5% BSA, 0.02% NaN_3 , 1 mM CaCl_2 , 1 mM MgCl_2 , and 1 mM ZnCl_2 . The bacteria were resuspended in 1.0 ml of the above buffer at 10^8 cells/ml. [^{125}I]-Labeled PSP (10^4 cpm) was added to the cells and incubated at 37°C or 23°C for 2 hr on an orbital shaker. The cells were pelleted by centrifugation, washed twice, and the radiolabel bound to the bacteria quantitated by a Beckman gamma counter. Specificity of PSP binding was determined by pre-incubation of the cells with 10 µg/ml of unlabeled PSP. Cation-

dependent binding was determined by incubation where one or all of the salts were altered in the incubation buffer.

Ligand blot assays were conducted as described previously (151). In brief, bacteria were lysed by sonication (4°C in PBS), and the membrane fraction collected following centrifugation. The membranes were sonicated a second time (in buffer containing 0.5% SDS), followed by centrifugation and the soluble material heated to 100°C for 5 min. Sample (the optical density adjusted to $A_{280} = 2.6$ units/ml: 10 µl (~0.1 units) was mixed with SDS containing sample buffer, separated on 10% polyacrylamide gel, and transferred to PVDF. The membrane was blocked using a modification of the method of Hossenlopp *et al.* (151). Briefly, the blot was incubated for 30 min in 10 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 0.02% NaN_3 , and 3% NP-40, 30 min in Tris buffer containing 1% BSA in place of detergent, and 30 min in Tris buffer containing 0.1% Tween-20. The membrane was incubated overnight in TBS containing 1% BSA, 0.1% Tween-20, and 10^5 cpm [^{125}I]-PSP. The blot was washed 3 times in TBS and exposed to X-ray film for 12 hr at -80°C using Kodak XAR-5 film. Specificity of PSP binding to bacterial proteins was determined by pre-incubation of the filter with unlabeled protein. Ligand binding blots were run on 3 separate occasions for reproducibility using 2 separate preparations of [^{125}I]-PSP from C3H/HeJ and BALB/c saliva.

Amylase Assay

The activity of human salivary amylase (SIGMA Chemical) was determined in the presence of varying concentrations of pure PSP, proline-rich protein (PRP), and BSA.

Amylase activity was determined by the method of Bernfeld using starch as a standard

substrate (133). Human amylase (1 μ g/ml) was resuspended in PBS. The incubation solution was comprised of 0.4 g soluble starch in 60 mM Tris-HCl containing 0.15M NaCl and 3 mM CaCl₂ and ZnCl₂. The stop solution consisted of 0.45% I₂, 0.045% KI and 0.03 N HCl. After termination of the reaction at 5, 10, 15, and 20 min, the enzyme activity was defined as the amount that hydrolyzed 1 mg starch/min/mg of protein. All values are expressed as mean $\pm$ S.E. for 3 separate determinations.

Results

Detection of PSP RNA Transcripts in Murine Lacrimal Gland

Total RNA derived from lacrimal tissue isolated from NOD, BALB/c and C3H/HeJ mice was assayed for the presence of PSP RNA transcripts by RT-PCR and Southern-blotting. As shown in Fig 14A, a strong PCR band was observed at 785 bp in lacrimal RNA of NOD but not C3H/HeJ or BALB/c mice. When hybridized with a PSP-specific internal oligonucleotide-probe, both NOD and BALB/c developed a band at the expected base pair size (Fig 14B) while the C3H/HeJ remained negative. Interestingly, the lack of PSP expression in C3H/HeJ mice is in concordance with previous reports by Hjorth et al (105) describing the parotid specificity of PSP gene expression using C3H/HeJ mice. Detection of the housekeeping gene, G3PDH, was used as a positive control to indicate that similar amounts of RNA was utilized in each PCR reaction (Fig 14C). G3PDH bands of 983 base pairs were present in all samples.

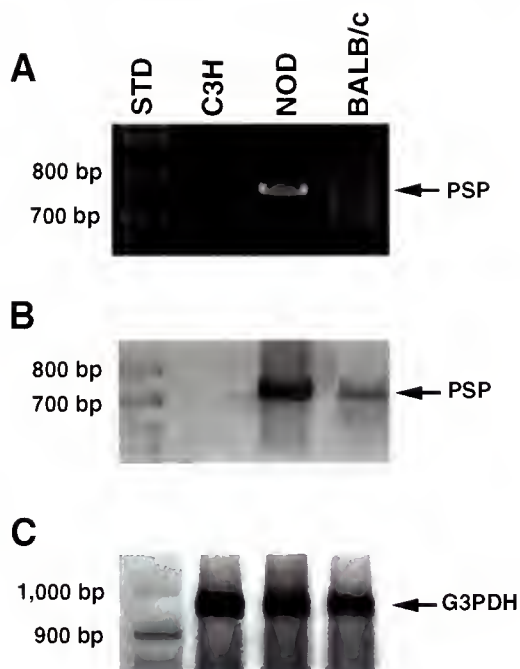


Figure 14 RT-PCR and Southern blot detection of PSP mRNA isolated from murine lacrimal glands. Total tissue RNA (2 μ g) was reverse-transcribed and resulting cDNA amplified by PCR using PSP-specific primers spanning the entire of length of the PSP transcript (785 bp). Panel A; Ethidium bromide-stained agarose gel containing PSP-amplified PCR product (10 μ l/lane) from 8 week C3H/HeJ (Lane 1), NOD (Lane 2), and BALB/c (Lane 3) lacrimal tissues. Panel B; Agarose gel (from Panel A) was transferred to a nylon membrane by Southern blotting and hybridized with a digoxigenin-labeled oligonucleotide probe specific for an internal PSP sequence. Blots were developed using an alkaline phosphatase labeled anti-digoxigenin antibody. Panel C; Positive control of RT-PCR and Southern Blot procedure using G3PDH-specific primers (983 bp product) and probe for C3H/HeJ (Lane 1), NOD (Lane 2), and BALB/c (Lane 3) RNA samples.

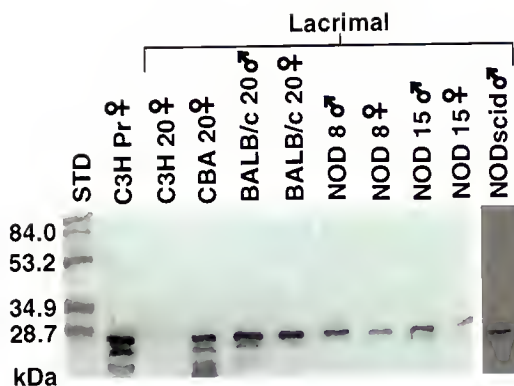


Figure 15 Western blot detection of parotid secretory protein in murine lacrimal glands. Total protein in pooled tissue lysates ($n=3$ mice/sample) from C3H/HeJ parotid gland (Lane 2; 1 $\mu\text{g}/\text{lane}$), was compared with pooled lacrimal gland lysates (30 $\mu\text{g}/\text{lane}$) from C3H/HeJ (Lane 3), CBA/J (Lane 4), BALB/c (Lanes 5 and 6), NOD (Lanes 7-10) and NOD-*scid* (Lane 11) mouse strains. Age (in weeks) and sex of sample groups are labeled above, with 15 week NOD mice being diabetic. Briefly, proteins were separated on 12% SDS-PAGE gels under reducing conditions, transferred to Immobilon P membranes, and incubated with rabbit anti-PSP antibody. Blots were developed with alkaline phosphatase-conjugated goat anti-rabbit antibody and substrate as per methods section. Prestained molecular weight markers are as follows; Bovine serum albumin, 84.0 kDa; Ovalbumin, 53.2 kDa; Carbonic Anhydrase, 34.9 kDa; and Soybean trypsin inhibitor, 28.7 kDa.

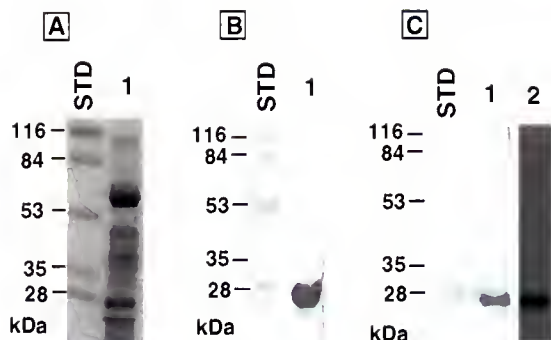


Figure 16 Purification of Parotid Secretory Protein. Four hundred μ l of total saliva was separated in a prep gel well (Panel A). Parotid secretory protein was identified by Western blot using antibody to PSP (Panel B) and cut from the gel. The purified protein was dialyzed, radiolabeled, and reanalyzed for purity by autoradiography and Western blot reactivity (Panel C lanes 2 and 3, respectively). Molecular weight standards (Bio-Rad) are Phosphorylase B, 116 kDa; Bovine Serum Albumin, 84 kDa; Ovalbumin, 53 kDa; Carbonic Anhydrase, 35 kDa; and Soybean Trypsin Inhibitor, 28 kDa.

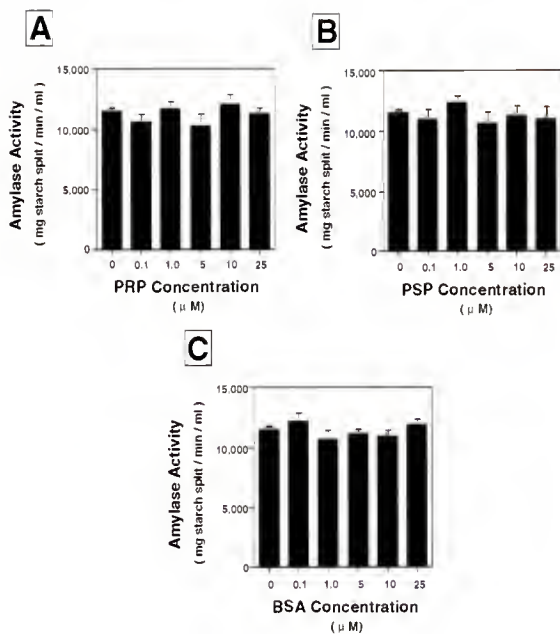


Figure 17 Amylase enzyme activity in the presence of saliva proteins. One μg of human saliva amylase was incubated in *in vitro* enzyme assays using starch as substrate. Amylase activity was assayed in the presence of increasing proline-rich protein (PRP; Panel A), PSP (Panel B) or BSA (Panel C) concentrations. All values represent the mean $\pm$ S.E. for 3 separate determinations performed in duplicate.

Detection of PSP in Lacrimal Gland Lysates

The transcribed and secreted PSP protein of mice has a molecular weight of 25.7 kDa when subjected to electrophoresis on 12% SDS-PAGE gels. Western blot analyses using an anti-PSP polyclonal antibody were used to investigate the presence of PSP protein in lacrimal tissues. As depicted in Fig 15, PSP bands were detected in the lacrimal glands of CBA/J, BALB/c, NOD, and NOD-*scid*, but not C3H/HeJ mice. There appeared to be inter-strain variability in the levels of PSP production with CBA/J > BALB/c > NOD > C3H/HeJ. Relative expression of PSP proved to be independent of age, however female mice displayed lower concentrations of PSP when compared with syngeneic males. Because C3H/HeJ mice did express PSP in their parotid glands, it is unknown why they fail to express PSP in their lacrimal glands.

Amylase Activity in the Presence of PSP

The coordinate synthesis and secretion of amylase and PSP into saliva implied a potential regulatory or chaperone function for PSP. Therefore, I examined the potential of several proteins including PSP, PRP and BSA (as a control) to modify amylase enzyme activity *in vitro*. PSP and PRP were isolated by preparative gel electrophoresis and checked for purity by staining with Coomassie Brilliant Blue and autoradiography of isolated PSP (Fig 16). As shown in Fig 17, PSP, PRP, and BSA had little, if any, influence on amylase activity.

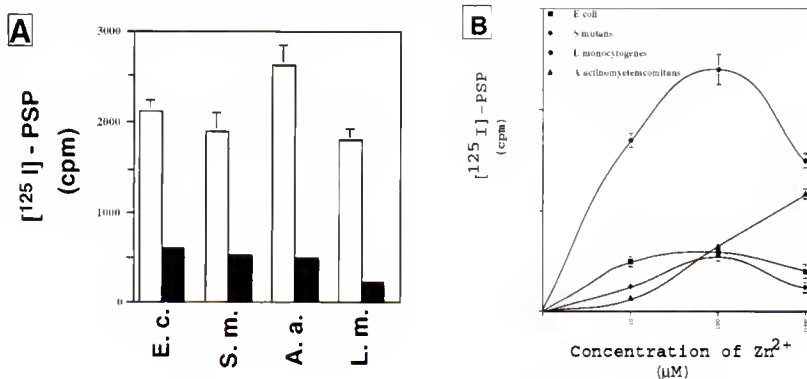


Figure 18 Bacterial binding of PSP. Panel A histogram represents radiolabeled PSP (10^4 cpm) incubated for 2 hr at 37° C with 10^8 bacterial cells/ml. [^{125} I]-PSP associated with the bacterial pellet was measured using a Beckman Gamma Counter. Solid bars represent pre-incubation with unlabeled PSP; open bars represent incubation with [^{125} I]-PSP only. Panel B, graph of PSP binding dependent on the presence of ZnCl in the incubation buffer for bacterial binding. All values represent mean $\pm$ S.E. for 3 separate determinations performed in duplicate.

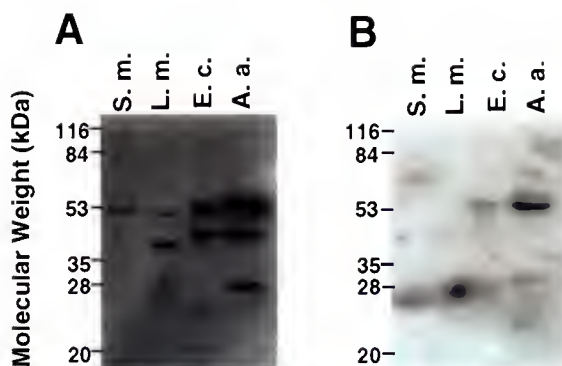


Figure 19 Autoradiograph of PSP binding to bacterial membrane proteins. Fifty µg of membrane protein was separated on a 10% polyacrylamide gel and transferred to PVDF membrane. The filter was incubated with 5×10^5 cpm [¹²⁵I]-PSP, washed and exposed to X-ray film. Panel A represents binding of C3H/HeJ PSP protein, however, similar results were obtained using radiolabeled BALB/c-derived PSP. Panel B represents a duplicate blot pre-incubated with 10 µg/ml unlabeled PSP for 2 hr prior to the addition of [¹²⁵I]-PSP. The binding assay was performed on 3 separate occasions for reproducibility.

Bacteria Binding of PSP

As one of the main functions of both saliva and tears is to serve as a non-immune barrier to bacterial infection from the external environment, PSP was examined for its ability to bind to oral or gastrointestinal tract pathogens. When radiolabeled PSP was incubated with intact bacteria (10^8 cells) in the presence of NaN_3 , binding ranged from 18-28% of the radiolabeled protein added (Fig 18, Panel A). Pre-incubation of bacteria with unlabeled PSP reduced the level of binding to 3-5% of the total counts added. The addition of NaN_3 to the incubation buffer metabolically kills bacterial cells, thus eliminating the potential for active uptake of PSP rather than strictly binding of PSP to the cell surface.

Since PSP is a leucine-rich protein, the potential exists for protein-protein interactions that are dependent on the presence of Zn^{2+} . To examine this potential, PSP binding to *E. coli*, *S. Mutans*, *A. actinomycetemcomitans*, and *L. monocytogenes* was evaluated with each of three cations (Ca^{2+} , Mg^{2+} , and Zn^{2+}) added separately to the binding buffer. As shown in Fig 18, Panel B, binding to intact bacteria was dependent on the presence of Zn^{2+} . Maximal binding occurred at 100 μM ZnCl_2 for all the bacteria except *A. actinomycetemcomitans* which showed maximal binding at 1 mM of Zn^{2+} cation. No binding above background was detected in buffer containing CaCl_2 or MgCl_2 (data not shown).

Specific Interaction of PSP with Bacterial Membrane Proteins

Binding of radiolabeled PSP to specific bacterial proteins was determined utilizing a ligand binding assay with solubilized membrane proteins. As shown in Fig 19,

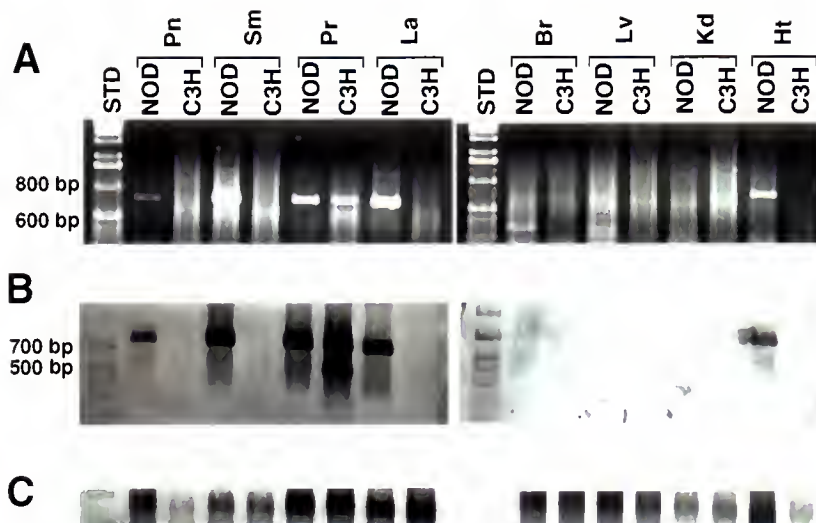


Figure 20 RT-PCR and Southern blot detection of PSP transcripts in NOD and C3H/HeJ tissues. Total tissue RNA from 8 wk NOD and C3H/HeJ mice was collected and amplified using PSP specific primers as previously described (see *Fig 1*). Panel A; Ethidium bromide-stained agarose gels containing PSP primer-amplified PCR product (10 μ l/lane) from NOD (odd numbered lanes) or C3H/HeJ (even numbered lanes) tissues. Tissues are labeled as follows: Pn, Pancreas; Sm, Submandibular; Pr, Parotid; La, Lacrimal; Br, Brain; Lv, Liver; Kd, Kidney; and Ht, Heart. Panel B; The gel from Panel A was transferred to nylon membrane by Southern blotting, hybridized with a PSP-specific, digoxigenin labeled oligonucleotide probe and developed with alkaline phosphatase-conjugated anti-digoxigenin antibody and substrate. Panel C. Positive control of RT-PCR and Southern blot procedure using G3PDH-specific primers (983 bp product) and probes for each RNA sample.

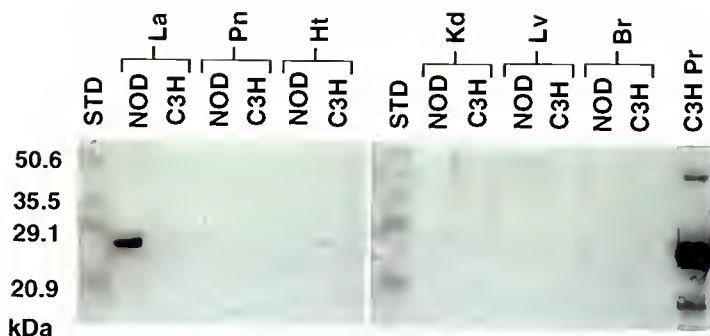


Figure 21 Western blot detection of parotid secretory protein in 8 week NOD and C3H/HeJ tissues lysates. Tissue protein (30 μ g/lane; parotid lane contained 1 μ g/lane) in pooled tissue lysates ($n=3$ mice/sample) from NOD (odd numbered lanes) and C3H/HeJ (even numbered lanes) mice were separated on 12% SDS-PAGE gels under reducing conditions, transferred to Immobilon-P membranes, and incubated with rabbit anti-PSP antibody. Blots were developed with alkaline phosphatase-conjugated goat anti-rabbit antibody and substrate. Similar result were obtained using diabetic NOD mice (15 weeks). Tissues are labeled as follows: Lc, Lacrimal; Pn, Pancreas; Ht, Heart; Kd, Kidney; Lv, Liver; and Br, Brain. Prestained molecular weight markers are as indicated in *Fig 7*.

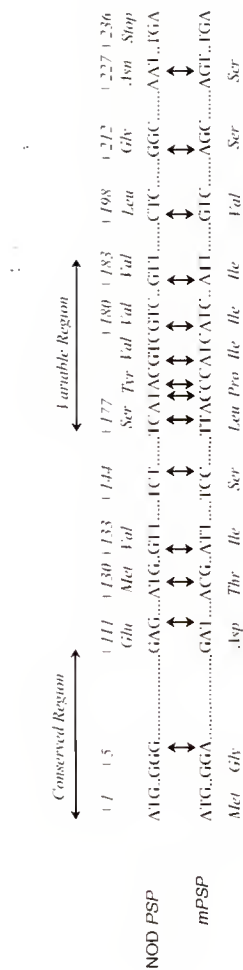


Figure 22 Sequence of parotid secretory protein cDNA derived from the parotid gland mRNA from the NOD mouse strain. PCR product obtained from PSP primer amplification of NOD parotid and lacrimal tissue RNA (see Fig 3) was eluted from agarose gels, purified, and sequenced. The sequences spanned from the 5' leader sequence and structural gene into the 3' untranslated region preceding the polyadenylation initiation site. The sequence comparisons are based on the published murine PSP cDNA sequence from Madsen et al (141). Only areas of nucleotide base pair differences between NOD PSP and the published sequence are shown, as well as any expected amino acid coding changes indicated by arrows.

membrane proteins from all four bacteria tested bound PSP. Binding of PSP to three distinct proteins was detected for all bacteria except *S. mutans* which contained a single PSP-binding protein at $M_r=53$ kDa. The other proteins ranged in size between 40-55 kDa. When the blots were pre-incubated with unlabeled PSP, the binding of radiolabeled PSP was either completely competed away (*S. mutans* and *L. monocytogenes*) or substantially reduced (*E. coli* and *A. actinomycetemcomitans*). The same bacteria binding pattern was obtained whether the source of PSP was from C3H/HeJ or BALB/c mice.

PSP Gene Dysregulation of the NOD mice

In addition to the lacrimal glands, a number of other organs and tissues were examined for the expression of PSP transcripts, including heart (atrial tissue), pancreas, submandibular glands, kidney, liver, and brain. Messenger RNA from each tissue was reverse-transcribed and the PSP and G3PDH genes amplified by PCR using specific primers. As shown in Fig 20, ethidium bromide staining revealed PCR bands in lacrimal, submandibular, parotid, pancreas, and heart, but not in brain, kidney or liver tissues of NOD mice. In contrast, only the parotid gland of C3H/HeJ mice yielded a visible PSP PCR band. Southern blot analysis (Fig 20B) confirmed the identity of the 785 bp bands as PSP. Note that weaker-staining bands of incorrect base pair size detected by ethidium bromide were not recognized by the PSP-specific probe. BALB/c mice displayed similar profiles to C3H/HeJ except that a band was detected in the lacrimal samples (data not shown). Positive control G3PDH bands were detected in all of the tissue samples (Fig 20C).

Western Blot Analysis of NOD and C3H/HeJ Tissues for the Presence of PSP

The above data indicated that PSP transcripts are expressed in the heart, pancreas, submandibular, parotid and lacrimal glands of NOD mice. For this reason, I analyzed by Western blotting a panel of tissue lysates derived from NOD and C3H/HeJ mice for the presence of PSP protein (Fig 21). In NOD mice, PSP protein was detected only in the parotid and lacrimal glands. In contrast, PSP was found only in the lysate of parotid glands from C3H/HeJ mice. Despite the presence of detectable mRNA transcripts in heart and pancreas of NOD mice, I was unable to detect the presence of PSP at the protein level.

Sequence Analysis of the NOD PSP Gene

The open reading frame of the PSP gene of NOD mice was sequenced using cDNA amplified from mRNA isolated from both the lacrimal and parotid glands. The PCR products were isolated from agarose gels and the full length DNA products sequenced by primer extension (Fig 22). As expected, the sequences of the lacrimal and parotid gland PCR products were identical, and revealed a 98% homology with the published murine PSP DNA sequence (14 base pair differences out of 705). Two regions, corresponding to amino acid residues 111-144 and amino acid residues 198-227, contain a few random mutations; however, two additional regions are of particular interest. The first, containing the first 110 amino acid residues, represents a highly conserved region where no amino acid changes were detected between the NOD and the published mouse PSP sequence. The second, containing 7 amino acid residues at position 177-183, is a highly substituted region in the NOD PSP gene sequence. These amino acid changes,

including a proline substitution at amino acid 178, could potentially cause major structural differences between the two PSP proteins.

Discussion

These results show that PSP is produced in the lacrimal glands of all normal mice tested, with the exception of C3H/HeJ, suggesting that this secretory protein is not restricted to the parotid and sublingual gland as first reported (105). The lack of expression in the lacrimal gland of C3H/HeJ mice supports the findings of Hjorth et al. (105), but raises further questions about the importance and/or functions of PSP as a secretory protein. These data suggest that although coordinately expressed with amylase, the protein does not influence enzyme activity (106). Further, detection of PSP in the lacrimal glands would indicate that the protein is not involved in saliva specific functions such as digestion. By far the most widely recognized function in saliva is that of growth control of microorganisms. The ability of PSP to bind to bacterial surface proteins in a Zn^{2+} dependent manner suggest that the protein may function as another host protein capable of modulating bacterial growth or colonization rates (152).

The sequence analysis of PSP mRNA of NOD mice revealed a number of amino acid differences in the carboxy-terminal region of the protein, suggesting the presence of strain-specific isoforms (141). Whether or not these changes influence the functional capacity is unknown; however, comparison of PSP with bacterial surface proteins for homologous leucine-rich regions revealed that potential leucine-rich interactions are present at the highly conserved amino terminal end of PSP. The observation of Zn^{2+}

dependence for bacterial interactions suggests that leucine-rich domains may be responsible for this property of PSP (153). This mechanism has been recently detailed for *L. monocytogenes* interaction with epithelial cell E-cadherin receptors leading to bacterial internalization (154).

Though these data clearly show that PSP is both transcribed and translated, in the lacrimal glands of most normal mice, the levels of PSP produced varied between strains. This inter-strain variability is consistent with earlier published data (105) which revealed that total levels of PSP and amylase were highly variable among mouse strains but that these two proteins maintained similar ratios in saliva (106). While studies by Shaw *et al.* (107,109) showed a concomitant appearance of PSP and amylase in the saliva of neonatal mice, this coincidental co-appearance of secreted protein was not controlled at the level of gene transcription since PSP, but not amylase, transcripts were detected in neonates at birth. Thus, gene expression of PSP and amylase in salivary tissue may be controlled by different and distinct transcriptional regulators. This may explain how PSP transcription occurs in the lacrimal tissues in the absence of amylase gene activation.

The presence of PSP transcripts in the submandibular gland, pancreas and heart of NOD mice is unique among the strains examined. However, PSP specific-primers spanning the cap to the untranslated region revealed only one PCR product, indicating that the novel PSP isoform which predominates aging NOD and NOD-*scid* mice saliva is most likely not attributed to alternate splicing events. Despite the presence of aberrant PSP transcription in several tissues of NOD mice possessing exocrine function, protein production was detected only in the submandibular, lacrimal and parotid glands, and not in the pancreas or heart. Furthermore, non-exocrine tissue (e.g. brain, liver, and kidney) did

not express PSP mRNA transcripts. One explanation is that only the salivary and lacrimal tissues possess both PSP transcripts and the necessary cytoplasmic factors for proper transcript processing and post-translational modification. Evidence for this was first described by Shaw and Schibler (107) who found abundant PSP transcription in the developing murine parotid gland at birth, yet translated protein was not detected until 12 days of age (155). This apparent lag between PSP gene expression and protein translation was attributed to rapid turnover of PSP transcripts in the cytoplasm.

Submandibular glands of neonatal mice produce PSP until about day 5, then gene expression and secretion subsides (108). Under normal conditions, cellular activity necessary for the production of PSP protein might still be present in adult submandibular glands, but PSP transcription would have ceased. Reappearance of PSP mRNA transcripts in the submandibular glands of NOD mice (121) in addition to the presence of appropriate cytoplasmic factors could explain why abnormal protein production of PSP is present only in the submandibular gland, but not the heart or pancreas. Whether this dysregulation observed in NOD mice is directly involved in the pathogenesis of the autoimmune activity and loss of immunological tolerance to the submandibular and lacrimal tissues or is merely a biological marker of generalized transcriptional alterations remains unknown and requires additional investigation.

In conclusion, these findings indicate that: first, PSP is normally expressed in the lacrimal glands of rodents, including NOD mice, where it potentially functions to control microbial growth or colonization; second, in addition to expression of PSP in lacrimal glands, NOD mice aberrantly express PSP transcripts (but not protein) in the pancreas and heart; and third, based on the cDNA sequence of PSP from NOD submandibular and

lacrimal glands, the PSP protein appears to contain a highly altered region which could result in strain-specific PSP isoforms.

CHAPTER 5

EVALUATION OF NOVEL PROTEOLYTIC ACTIVITY DETECTED IN THE SALIVA OF AGING NOD MICE

Introduction

Results in this dissertation suggest that PSP is enzymatically cleaved in older NOD saliva. The cleavage occurs between leucine and asparagine at the 26th and 27th amino acids of the protein. Database searches did not reveal any known enzymes which may be responsible for this cleavage. However, since PSP cleavage corresponds to the time of dramatic acinar cell loss in the NOD mouse, I investigated the potential of both matrix metalloproteinases (MMPs) and apoptotic cysteine proteases to cleave PSP through bystander activity. These enzymes, involved in glandular restructuring and apoptotic cell death, respectively, may play a key role in the loss of submandibular acini in NOD and NOD-*scid* mice. Therefore, a novel proteolytic activity detected in aging NOD and NOD-*scid* saliva may be indicative of glandular trauma as well as further establishing novel protein expression in the exocrine glands.

In this specific aim, proteolytic activity in NOD saliva was evaluated for the ability to cleave purified PSP or PSP from control mice. In addition, since programmed cell death appears independently of lymphocyte infiltration of the submandibular gland, I investigated the potential activation of apoptotic proteases in the saliva and salivary gland lysates of NOD mice. The best characterized of the apoptotic cysteine proteases is the

interleukin-1 β converting enzyme (ICE) which is required for induction of apoptosis through the CD95 (FAS) signaling pathway (119). In this specific aim, increases in cysteine protease activity were detected by both an *in vitro* activity assay and Western blot analyses of saliva and submandibular, but not parotid glands, from NOD and NOD-*scid*, as compared to BALB/c and young NOD mice. However, the ability of saliva and both submandibular and parotid gland lysates to generate the aberrant NOD PSP cleavage product suggest that additional cellular proteases are activated in these tissues as well.

Zymogram analysis was used to demonstrate that unique MMP activity is present in the saliva and gland lysates from NOD mice. These proteases are responsible for the breakdown of extracellular matrix components and may contribute to glandular restructuring in NOD mice (111). In addition, the ability of NOD saliva to cleave BALB/c PSP was inhibited by the presence of EDTA. EDTA is a metal ion chelator used as a ubiquitous inhibitor of MMP activity through its ability to sequester zinc ions necessary for MMP proteolytic activity (111). EGTA, a calcium chelator, was unable to inhibit PSP cleavage. Together, these findings confirm that the aberrant cleavage of PSP is due to unique protease activity in NOD saliva. While the protease remains unknown, the detection of both a unique MMP activity in NOD saliva and EDTA inhibition suggest that MMP activity may be involved in the cleavage of PSP.

Materials and Methods

Materials

BALB/c and NOD/UF mice were bred and maintained under SPF conditions in the mouse facility of the Department of Pathology and Laboratory Medicine at the University of Florida, Gainesville, FL. NOD-*scid* mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Both male and female mice at ages 8 weeks and 20 weeks of age were used. NOD mice were routinely tested for blood glucose levels using Chemstrip bG reagent strips (Boehringer Mannheim, Indianapolis, IN). Consecutive elevated fasting blood glucose levels >240 mg/dl were considered onset of diabetes, after which the mice were maintained on daily insulin injections. NOD mice at 20 weeks of age were separated into diabetic and pre-diabetic groups for protease assays.

Antibody for rat cystatin (156) was a generous gift from Dr. Gurrinder Bedi, Magainin Pharmaceuticals, Plymouth Meeting, PA. Pilocarpine, d,l-Isoproterenol, phenyl-methylsulfonyl fluoride (PMSF), dithiothreitol (DTT), dimethyl sulfoxide, papain, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin, and sodium benzoyl-dl-arginine-*p*-nitroanilide (BAPNA) were obtained from SIGMA Chemical Co. (St. Louis, MO). Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad (Richmond, CA).

Saliva Collection and Preparation of Gland Lysates

Saliva was collected from control and experimental groups of mice following stimulation of secretion using isoproterenol (0.20 mg/100 g body weight) and pilocarpine

(0.05 mg/ 100g body weight) dissolved in saline. The secretagogue cocktail was injected (0.1 ml volume) intraperitoneally, with saliva subsequently collected from the oral cavity by micropipet and placed into chilled 1.5 ml microfuge tubes (67). Saliva samples were collected from groups of 6 mice and then frozen at -80°C until analyzed for temporal changes by enzyme assay, SDS-polyacrylamide gels, and Western blotting.

Parotid and submandibular glands were excised from mice killed by cervical dislocation. Each gland was separated from connective tissue, fat, and lymph nodes, then homogenized in 10 mM Tris buffer (pH 7.4) and immediately frozen at -80°C. Protein assays of both saliva and gland lysates were performed using the method of Bradford (132) with bovine serum albumin as the standard.

Cysteine Protease Assays

Protease activity in saliva and gland lysates were determined using a standard protease assay as described elsewhere (157, 158). The assay relies on the cleavage of the chromogenic reagent, BAPNA. The incubation buffer consisted of 25 μ l 100 mM BAPNA in DMSO, 10 μ l unknown sample, 190 μ l PMSF buffer consisting of 0.2 mg/ml DTT, 0.5 mg/ml Na₂EDTA and 1.0 mM PMSF in 100 mM phosphate buffer (pH 6.0). Experimental samples, as well as a dilution profile of papain were incubated at 37°C for one hr. The reactions were terminated by the addition of 25 μ l of glacial acetic acid, adjusted to 1.0 ml with ddH₂O, and the optical density determined at OD₄₀₅ nm to determine the amount of *p*-nitroaniline released. A standard curve was generated from the papain. All values were expressed for the mean $\pm$ standard error for all samples performed in duplicate on three separate occasions.

Polyacrylamide Gel Electrophoresis, Zymogram, and Western Blot Analysis

Total salivary proteins (15 µg/well) or gland lysates (50 µg/well) were subjected to electrophoretic separation on a 1.5-mm-thick 10% or 12% SDS-polyacrylamide gel using the modified Tris-glycine system of Pugsley and Schnaitman (136). For zymogram gels, 3% gelatin was added to the gel mix prior to casting. Following electrophoresis, the proteins were transferred for 2 hr at 70V to Immobilon-P membranes (Millipore, Bedford, MA) for Western Blotting or washed and incubated for 24 hr in Tris buffer with zinc for zymogram analysis (138). Polyclonal antibodies specific for murine ICE and Nedd-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and Apopain/CPP 32 (UBI, Lake Placid, NY) were used to detect specific apoptotic proteases at dilutions specified by the manufacturer. Polyclonal rabbit anti-rat cystatin (1:500 dilution) was incubated with each membrane for 12 hr at 25°C. The blocking buffer consisted of 3% nonfat dry milk and 3% BSA in Tris-buffered saline. Saliva from chronically isoproterenol-treated rats was used as a positive control for assessing the performance of the anti-cystatin antibody, while pre-incubation of the anti-cysteine protease antibodies with the specific peptide antigen served to determine the specificity of the antibody-antigen reactions. Following three 10-min washes, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and exposed to substrate as previously described (121).

Proteolytic Digestion of PSP

For assays using radiolabeled, purified PSP, 10 μ l saliva or 30 μ g salivary gland lysates (total protein) was incubated with 10^4 cpm of [^{125} I]-PSP in a 50 μ l total volume for 4-6 hr at 37°C. The digestion was stopped by the addition of SDS-PAGE sample buffer and heating to 100°C for 5 min prior to electrophoretic separation on a 12% SDS-polyacrylamide gel as described above. The gels were dried and exposed to Kodak XAR-5 X-ray film for 48 hr. For saliva mixing assays, equal volumes of NOD or BALB/c saliva (2.5 μ l) were diluted up to a total volume of 20 μ l in PBS and incubated at 37°C for 2-6 hr. The addition of EDTA or EGTA to a final concentration of 1mM to 100mM was used to assess proteolytic digestion upon removal of cations. The reaction was stopped by the addition of 5 μ l of SDS-PAGE sample buffer and heated to 95°C for 5 minutes. Electrophoresis and PSP Western blot detection were conducted as described above.

Statistical Analysis

All measures of variance are given as standard deviations of the mean. Tests of significance for differences between independent means were performed with the unpaired Student *t* test. Results in which $P < 0.05$ were considered significant.

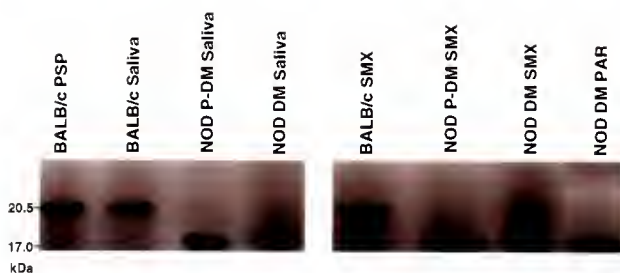


Figure 23 Autoradiogram of differential PSP migration following incubation with saliva or salivary gland lysates. 10^4 cpm of [^{125} I] PSP purified from BALB/c saliva was incubated for 4-6 hours at 37°C with whole saliva, submandibular (SMX) lysates, or parotid (PAR) lysates from BALB/c or NOD mice. NOD P-DM, prediabetic NOD; NOD DM, diabetic NOD. Radiolabeled BALB/c PSP (Lane 1) incubated in PBS served as control migration.

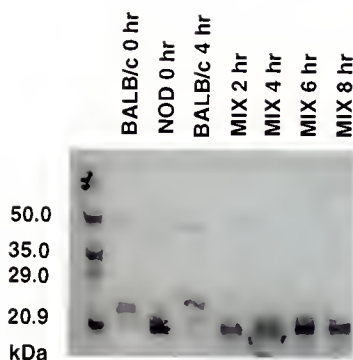


Figure 24 Western blot of differential PSP migration following incubation of NOD and BALB/c saliva. Saliva (2.5 μ l) from BALB/c (0 hr incubation; Lane 2), NOD (0 hr incubation; Lane 3), BALB/c (4 hr incubation; Lane 4) was separated on a 10% SDS-PAGE gel and immunoreacted with anti-PSP. Lanes 5-8 depict equal volumes of BALB/c and NOD saliva (2.5 μ l) mixed and incubated in 15 μ l PBS for 2hr, 4 hr, 6 hr, and 8 hr, respectively. Molecular weight standards are as follows: bovine serum albumin, 84000 Da; ovalbumin, 54000 Da; carbonic anhydrase, 35000 Da; and soybean trypsin inhibitor, 28000 Da.

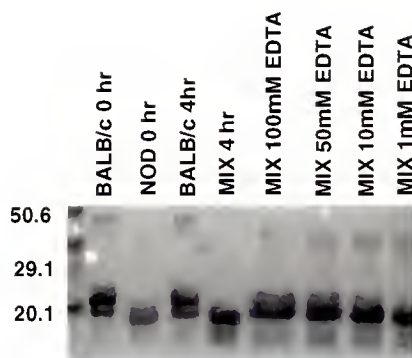


Figure 25 Western blot depicting EDTA inhibition of the proteolytic cleavage of PSP. Saliva (2.5 μ l) from BALB/c (0 hr incubation; Lane 1), NOD (0 hr incubation; Lane 2), BALB/c (4 hr incubation; Lane 3) was separated on a 10% SDS-PAGE gel and immunoreacted with anti-PSP. Lanes 4-8 depict equal volumes of BALB/c and NOD saliva (2.5 μ l) mixed and incubated in 15 μ l PBS with 0, 100, 50, 10, and 1 mM concentrations of EDTA, respectively. Molecular weight standards are as described in Fig 24.

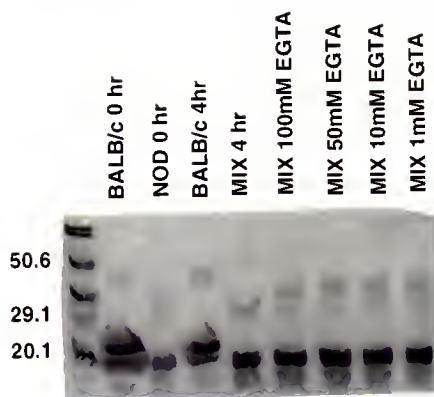


Figure 26 Western blot depicting proteolytic cleavage of PSP in the presence of EGTA. Saliva (2.5 μ l) from BALB/c (0 hr incubation; Lane 1), NOD (0 hr incubation; Lane 2), BALB/c (4 hr incubation; Lane 3) was separated on a 10% SDS-PAGE gel and immunoreacted with anti-PSP. Lanes 4-8 depict equal volumes of BALB/c and NOD saliva (2.5 μ l) mixed and incubated in 15 μ l PBS with 0, 100, 50, 10, and 1 mM concentrations of EGTA, respectively. Molecular weight standards are as described in Fig 24.

Table 5. Cysteine Protease Activity in Saliva

	ACTIVITY*			
	$\mu\text{g/ml}$		$\mu\text{g/mg protein}$	
	8 week	20 week	8 week	20 week
BALB/c	15.2 $\pm$ 2.6	16.7 $\pm$ 2.7	15.9 $\pm$ 2.7	12.8 $\pm$ 2.1
NOD P-DM	14.8 $\pm$ 1.9	45.8 $\pm$ 5.3	10.3 $\pm$ 1.9	39.2 $\pm$ 4.8
NOD DM	N.A. [†]	40.3 $\pm$ 6.8	N.A. [†]	39.5 $\pm$ 3.7
NOD-scld	12.1 $\pm$ 3.2 [‡]	67.5 $\pm$ 12.3	13.6 $\pm$ 2.2 [‡]	67.0 $\pm$ 4.7

* All values expressed as the mean $\pm$ standard error for n=6 animals/group. The assays were performed in duplicate on three separate occasions.

[†]N.A.= not available due to the later age of diabetes onset in the University of Florida NOD colony.

[‡]n=2

Results

Generation of the Aberrantly Processed 17kDa PSP Isoform

As presented in Chapters 2, 3, and 4, NOD and NOD-*scid* mice abnormally express PSP in their submandibular glands and, that with increasing age, this PSP is aberrantly processed through enzymatic digestion in their submandibular glands (121). Using radiolabeled PSP purified from BALB/c saliva, I assessed the ability of saliva and gland lysates to enzymatically digest PSP to the aberrant isoform. Incubation of BALB/c-derived ¹²⁵I-labeled PSP with NOD saliva resulted in the digestion of the 20.5 kDa PSP isoform to the 17.0 kDa PSP within 4 to 8 hr incubation at 37°C. Incubation for more than 8 hr caused a complete degradation of the radiolabeled PSP (data not shown). Incubation of PSP with BALB/c saliva or submandibular gland lysates failed to generate the 17.0 kDa PSP isoform (Fig 23), and there was no evidence for partial degradation of PSP by incubation of the protein at 37°C in PBS. Also shown in Fig 23, submandibular gland lysates from both 20 week old pre-diabetic and diabetic NOD mice were able to cleave the BALB/c PSP to the aberrant 17.0 kDa isoform.

In addition to assays using radiolabeled PSP, PSP cleavage was demonstrated through the mixture of pooled 18wk NOD and BALB/c saliva. Since NOD saliva contained only the cleaved PSP isoform while BALB/c contained only native PSP, cleavage of BALB/c PSP could be differentiated by shifts in the molecular weight as the protein was digested. As shown in Fig 24, complete digestion of PSP in BALB/c saliva was achieved following a 2 hr incubation. BALB/c saliva in the absence of NOD saliva failed to generate the cleaved PSP isoform after 4 hr of incubation.

Inhibition of PSP Digestion with EDTA

To test the ability of divalent cation chelators to inhibit the proteolytic digestion of PSP, NOD and BALB/c saliva were incubated at 37°C for 2 hr in the presence of EDTA or EGTA. As shown in Fig 25, digestion of BALB/c PSP was inhibited by 100, 50, and 10 mM final concentrations of EDTA. Inhibition was greatest at higher EDTA concentrations and was dose-dependent. At 1 mM EDTA concentration, the majority of BALB/c PSP was cleaved. No inhibition was detected upon the addition of 100, 50, 10, or 1 mM EGTA to the saliva mixture (Fig 26). This indicates that the presence of divalent metal ions, but not calcium ions, is necessary for proteolytic cleavage of PSP.

Elevated Cysteine Protease Activity in NOD Mice

Changes in cysteine protease activity in the saliva of NOD and BALB/c control mice was assessed to correlate changes in the histology with potential apoptosis of acinar cells as observed previously (121). As presented in Table 5, there was an approximate 3-fold increase in cysteine protease activity in the saliva of 20 week old pre-diabetic and diabetic NOD mice when compared to age-matched BALB/c controls or 8 week old NOD animals ($P < 0.01$). In NOD-*scid* mice, this increase in proteolytic activity was even greater (67.5 vs. 16.7; $P < 0.005$). Saliva concentrations of cysteine protease activity ranging between 14-17 $\mu\text{g/ml}$ saliva were measured in BALB/c, pre-diabetic NOD and NOD-*scid* mice 8 weeks of age. The same trends in relative protease activity in saliva did not vary greatly if proteolysis was calculated relative to constant protein rather than to constant volume (Table 5). There were no differences in the levels of cysteine protease activity

between 8 and 20 wks of age in BALB/c animals or between male and female mice ($P>0.05$).

To further define the source of the increased proteolytic activity in saliva, both parotid and submandibular gland lysates were prepared from 20 week old mice. Parotid gland lysates from BALB/c, 8 wk old prediabetic (NOD P-DM) and diabetic NOD (NOD-DM) as well as NOD-*scid* mice showed similar levels of proteolytic activity, approximately 15 μg protease/mg total gland lysate protein (Fig 27; $P>0.05$). In contrast, gland lysates prepared from the submandibular glands of older mice revealed an increased cysteine protease activity in pre-diabetic NOD mice that was even greater with onset of diabetes (37.3 vs. 78.8 μg protease/mg gland protein, respectively; $P<0.01$). In submandibular gland lysates of NOD-*scid* mice, proteolytic activity proved to be the greatest, correlating with previous histological analyses and suggesting loss of acinar cells through cell death (121).

Detection of Elevated ICE, Nedd-2, and Apopain/CPP 32 in NOD Saliva and Gland Lysates.

Saliva and gland lysates were evaluated by Western blot to confirm the increased activity of apoptotic cysteine proteases in NOD mice. As presented in Fig 28, whole saliva possessed an increase in immunoreactive material for ICE, Nedd-2 and Apopain/cpp32 in older NOD and NOD-*scid* mice. Potential pro-enzyme forms ($M_r=35\text{-}50\text{ kDa}$) were detected in saliva from 8 and 20 wk NOD as well as the NOD-*scid* animals, but not in BALB/c mice. The active subunits detected by the antibodies were: ICE (p10), Nedd-2 (p12) and Apopain (p12) (25-27). Thus, the different mobilities of the proteins

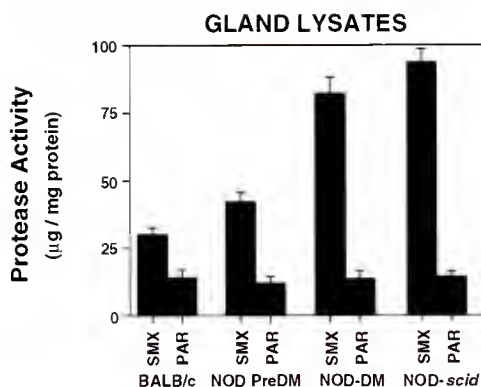


Figure 27 Histogram of cysteine protease activity in salivary gland lysates. Parotid (PAR) and submandibular (SMX) gland lysates were incubated with chromagenic substrate, BAPNA, for 60 min at 37° C. Standard curve was generated by linear regression analysis of papain digestion of BAPNA. All values represent the mean $\pm$ S.E. performed in duplicate on three separate occasions.

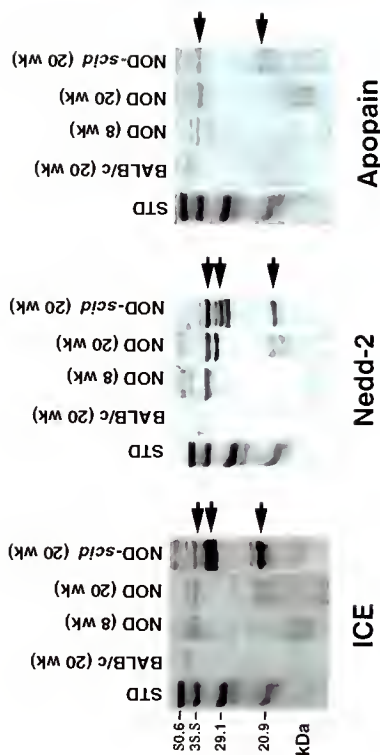


Figure 28 Western blot analysis of saliva for the presence of apoptotic proteases. Whole saliva (15 μ g) was separated on a 12% SDS-PAGE and evaluated for the presence of murine ICE, Nedd-2 and Apopain/CPP 32 using rabbit polyclonal antibodies. The fidelity of the antibody reaction was established by pre-incubation of the antibodies with the peptide antigen (1 μ g/ml) prior to reaction with the nitrocellulose membrane. Cleavage of the ICE p45 pro-enzyme generates p20 and p10 active subunits. Proteolytic cleavage of Nedd-2 the p50 pro-enzyme generates the p17 and p12 subunits of the mature protease. of the Apopain/CPP 32 p35 pro-enzyme produces the p17 and p12 subunits of the mature protease. Prestained molecular weight standards (Bio-Rad) are as in Fig 24.

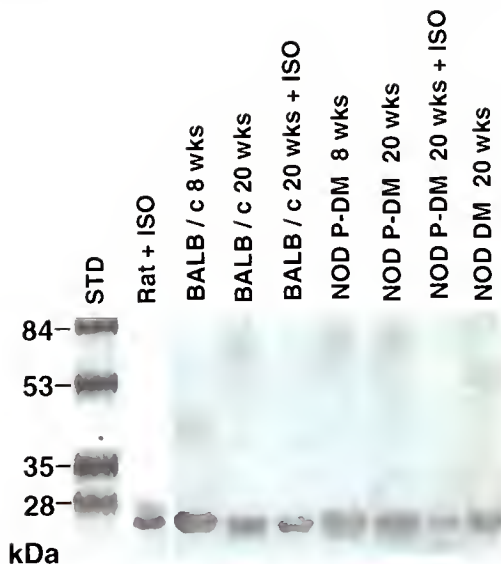


Figure 29. Western blot analysis of saliva for the presence of the cysteine protease inhibitor, cystatin. Whole saliva (15 μ g) was examined on a 12% SDS-PAGE gel using a rabbit-polyclonal anti-rat cystatin. A chronically-treated isoproterenol rat whole saliva was included as a positive control. Isoproterenol treatment of mice was 2X ip. daily for 3 days. P-DM, prediabetic NOD; DM, diabetic NOD. Molecular weight standards are as follows: bovine serum albumin, 84000 Da; ovalbumin, 54000 Da; carbonic anhydrase, 35000 Da; and soybean trypsin inhibitor, 28000 Da.

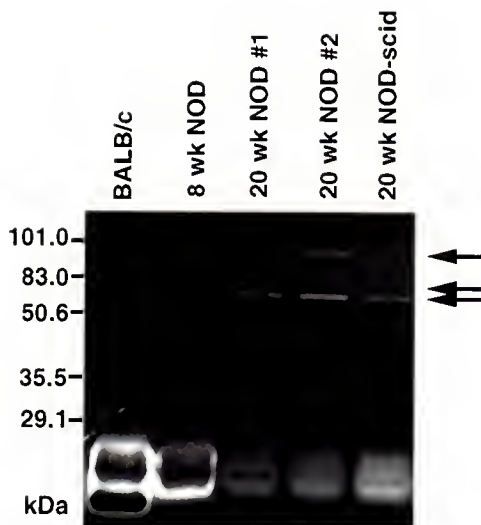


Figure 30 Zymogram gel showing distinct MMP activities in NOD and BALB/c saliva. Whole saliva (3 μ l) from BALB/c (Lane 1), 8 wk NOD (Lane 2), 20 wk NOD (Lane 3), diabetic NOD (Lane 4), and NOD-*scid* (Lane 5) was separated on a 10% SDS-PAGE gel containing 3% gelatin. The gel was then washed to remove SDS, incubated for 24 hr in Tris buffer containing Zn^{2+} 24 hr, and stained with Coomassie Blue. Arrows corresponding to unique zones of clearance in NOD samples indicate the presence of gelatinase activity.

recognized in the gel could be non-dissociated complexes of the active apoptotic protease subunits or digestion products from interaction with proteases from saliva. The antibodies failed to react with material in gland lysates, potentially due to their low concentration in total protein preparations. As a control for the proper identification of cysteine proteases in saliva, the same preparations were reacted with primary antibody pre-incubated with the peptide antigen supplied by the manufacturers. Under these conditions, only the highest molecular weight band ($M_r \approx 55$ kDa) was detected with the addition of secondary antibody and chromagenic substrate.

Determination of Cystatin Levels by Western blot.

Cystatins are a series of cellular proteins produced by various tissues to limit proteolytic activity in response to injury (158). Synthesis of these proteins can be induced in salivary glands by chronic treatment with the β -adrenergic agonist, isoproterenol (156). Saliva was analyzed for the levels of cystatin by Western blotting using a polyclonal antibody to rat cystatin. As presented in Fig 29, a protein of approximately 15 kDa that migrated similarly to the cystatin protein present in whole rat saliva isolated following chronic isoproterenol treatment, was observed in each saliva tested. There were no differences in the levels of cystatin detected in saliva from BALB/c or NOD mice irrespective of age. Interestingly, chronic treatment of mice with isoproterenol appeared to decrease the levels of cystatin present (Fig 29). This was reflected in a BAPNA digestion assay where proteolytic activity increased from 14 to 31 μg protease/ml and 43 to 51 μg protease/ml in BALB/c and pre-diabetic NOD mice, respectively ($P < 0.05$).

Detection of Novel MMP Activity in NOD Saliva and Gland Lysates

To evaluate the presence of matrix metalloproteinase activity in NOD exocrine tissues, gland lysates or saliva from NOD, NOD-*scid*, or BALB/c mice were analyzed by zymogram gel. As shown in Fig 30, unique zones of gelatin clearance were detected at approximately 98 kDa and 60 kDa in NOD and NOD-*scid* gland lysates. High levels of MMP activity were detected at 55 kDa in BALB/c mice, while lesser activity at this molecular weight was present in NOD strains. Gelatin clearance was detected at ~20 kDa in all samples; however, this activity was weakest in 20 wk NOD mice. Total MMP activity in NOD and BALB/c tissue lysates was found to be similar by azocoll chromagenic assay (data not shown). These findings confirm that active MMPs are present in both NOD and control BALB/c mice, although, unique MMP activities are detected only in NOD mice.

Discussion

The results presented in this aim confirm that the appearance of the cleaved PSP isoform in the saliva of aging NOD and NOD-*scid* mice is due to unique proteolytic processing. While the protease responsible for this enzymatic activity is unknown at this time, the inhibition of PSP cleavage by the addition of EDTA, but not EGTA, suggests that the presence of metal ion, but not calcium, is necessary for this activity. The matrix metalloproteinases (MMPs) are a class of zinc-dependent enzymes which, in conjunction with their specific inhibitors (TIMPS), are responsible for restructuring and maintenance

of the extracellular matrix (110). Over 12 MMPs have been described and include the collagenases, gelatinases (collagen type IV), stromelysins (laminases), and elastins which are able to cleave virtually all components of the extracellular matrix (111). Expression of specific MMPs during the developmental process is responsible for resculpturing the extracellular environment and thereby dictating cellular turnover and differentiation. Since MMP activity is highly dependent on the presence of Zn^{2+} , EDTA is commonly used as a potent inhibitor of MMP activity (110). Inhibition of MMP activity by EDTA is typically detected at 4 mM concentrations. In the present studies, a dose dependent inhibition of PSP digestion was detected between 100 and 1 mM EDTA concentrations. Interestingly, maximal inhibition by EDTA was detected at 10 mM, but not 1 mM, EDTA concentrations. This indicates that both MMPs and the enzyme responsible for PSP cleavage are inhibited by similar concentrations of EDTA. Furthermore, unique MMP activity was detected in the saliva and gland lysates of NOD mice that was not detected in BALB/c mice. These results suggest that novel MMP activity in NOD exocrine tissues remains a strong candidate for PSP cleavage.

Cellular homeostasis depends on regulated cell proliferation coupled to cell death. Extracellular signaling molecules are capable of regulating glandular cell populations through a series of intracellular events termed programmed cell death or apoptosis. The cytokine tumor necrosis factor (TNF) and the related protein CD95 (FAS) are immune system molecules capable of triggering apoptosis. Programmed cell death is mediated intracellularly by a proteolytic cascade involving members of the cysteine protease family which cleave important cellular proteins including pro-enzymes of other members of this class of proteases (115). A prototype protein of the cysteine proteases is interleukin-1 β -

converting enzyme (ICE) which has been characterized as the activator of the cytokine interleukin-1 β through cleavage of its precursor at Asp116/Ala117 (120). All members of the ICE-related cysteine protease family cleave their substrates after an aspartate residue followed by a small amino acid residue which is important for substrate consensus recognition (120). Therefore, high levels of cysteine protease activity may be indicative of both the activation of apoptotic mechanisms as well as the processing of proinflammatory cytokine precursors.

In the present study, I have found that NOD and NOD-*scid* mice had high concentrations of cysteine protease and MMP activity in their saliva and submandibular gland lysates, consistent with the histological observations suggestive of submandibular acinar cell death. However, the parotid glands from NOD mice, which do not show evidence of a strong autoimmune attack, possessed similar levels of cysteine protease activity as that seen in tissue from BALB/c control mice. The increased cysteine protease activity in the submandibular glands correlated with the time at which autoimmune leukocytes generally appear in the glands and subsequently display secretory dysfunction. The greatest level of protease activity was present in the NOD-*scid* mice despite the absence of functional B- and T-lymphocytes. Autoimmune lymphocytes are not, therefore, the main underlying factor for increased acinar cell turnover. The increased cysteine protease activity in NOD lysates did not appear to be the result of a loss in the control of synthesis of the specific inhibitor, cystatin, as evidenced by similar saliva protein levels in BALB/c and NOD mice. Thus, the detection of cysteine protease activity in the submandibular gland is consistent with the concept that NOD mice possess a genetic

predisposition for glandular loss of function leading to activation of the apoptotic pathway and subsequent activation of the immune system (121).

While parotid glands of aging NOD mice are able to generate the aberrant PSP 17.0 kDa isoform, levels of cysteine protease activity are similar to that of BALB/c animals. The further observation that the unique cleavage site of PSP (Asn26/Leu27) is not an amino acid consensus sequence for cysteine proteases, suggests that aberrant proteolytic processing of PSP in NOD mice is independent of the activation of this class of enzymes.

Considered as a whole, these studies are painting a complex picture as to the physiological state of the salivary glands of NOD mice. Reports (107, 108) indicate that PSP is synthesized in the neonatal submandibular gland acinar cells of normal mice up to 5 days of age after which time synthesis ceases, while synthesis in the parotid gland continues. In the NOD mouse, the reappearance of transcripts and translated PSP in the submandibular gland suggest that there is a breakdown in cell differentiation and re-expression of developmental proteins. This loss of developmental control must extend to the parotid gland with the subsequent abnormal processing of PSP in both glands and may be dependent on aberrant MMP activity. The more extensive glandular disruption of the submandibular gland could potentially activate programmed cell death in the acinar cells leading to the observed histopathology seen in the NOD-*scid* background. The resulting cell death may then contribute to the autoimmune pathology through the activation of immune system activators and effectors such as cytokines and the generation of autoantibodies which exacerbate the glandular dysfunction leading to loss of secretory

function. The end result of this process on exocrine tissues would be clinical presentations of xerostomia and xerophthalmia.

CHAPTER 6

CONCLUSION AND FUTURE DIRECTION

It is generally believed that the physiological and biochemical changes taking place in the exocrine glands of Sjögren's Syndrome patients and animal models of this disease are a direct result of the lymphocytic attack on the exocrine tissues. For this reason, most research endeavors have focused on the role of T and B lymphocytes, autoantibodies, and cytokines in the pathogenesis of anti-exocrine autoimmunity. These studies have overwhelmingly shown that strikingly similar lymphocyte phenotypes and cytokine expression are detected in the salivary and lacrimal glands. In the present study I have demonstrated that, in the scope of the markers tested, these features of the anti-exocrine response in NOD mice resemble those detected in patient biopsies and other disease models. Since normal salivary flow rates are detected in immunocompromised NOD-*scid* mice, there is little doubt that the lymphocytic component is crucial for the loss of secretory function in NOD mice. Therefore, this work confirms the NOD mouse as a valid model for the study of autoimmune-induced sicca syndrome.

Of considerable interest is the fact that, while numerous animal strains (i.e. MRL/lpr, NZB/NZW) develop lymphocytic foci in the exocrine tissues, only the NOD mouse exhibits a loss of secretory function similar to that of human patients. This suggests that, similar to the observation of insulinitis in non-diabetic NOD mice, the mere presence of the lymphocytes in the exocrine tissues is not sufficient for the loss of

glandular function. Therefore, the immunological and physiological events occurring in the exocrine tissues of NOD mice must be unique from those taking place in other animal models displaying sialadenitis. Comparing the similarities and differences between sialadenitis in these animal models should provide insight into the autoimmune effector mechanisms which result in secretory dysfunction.

Focal lesions of the submandibular glands of NOD mice cover only a small portion of the total tissue area, and the parotid glands do not develop extensive lymphocytic infiltration. Furthermore, the parotid glands of normal mice are able to compensate for the surgical removal of the submandibular glands by increasing secretory flow to volumes comparable to that seen prior to surgery. Despite this fact, nearly 90% of secretory function is lost in NOD mice between 8 and 20 weeks of age. Therefore, both submandibular and parotid gland function is affected by the autoimmune processes. These findings further suggest that direct lymphocyte-mediated cell killing is not the principle effector mechanism for the loss of secretory function.

Cytokines such as IFN γ and TNF α which are prevalent in the focal lesions of human labial lip biopsies and exocrine tissues of animal models have been studied for their potential cytotoxic properties towards exocrine cells. For example, *in vitro* studies have shown that IFN γ and TNF α are cytotoxic to human salivary duct cell lines, hinting that similar events may be taking place *in vivo*. In the parotid glands of NOD mice, little or no mRNA transcripts for IFN γ are detectable by RT-PCR. Furthermore, the presence of large quantities of these cytokines in the exocrine tissues of MRL/lpr and NZB/NZW mice which do not lose secretory function strongly indicates that bystander cytotoxic activity of these cytokines is not sufficient to cause the loss of secretory function. It should be noted

that this does not exclude a potential role of cytokine cytotoxicity in mediating secretory dysfunction in NOD mice by other, as yet unidentified, contributing factors.

The finding of autoantibodies directed against the β -adrenergic and muscarinic receptors provides a plausible explanation for the loss of both parotid and submandibular secretory function, as antibodies in the sera could influence function of either tissues. These cell surface receptors are down-regulated in the salivary glands of NOD mice, which may indicate the mechanism by which these autoantibodies could contribute to secretory dysfunction. To confirm the pathogenic role of these antibodies is an area of intense interest. Researchers have yet to evaluate the presence of similar autoantibodies in human sera, and the role of autoantibodies in Sjögren's Syndrome is unclear. The recently developed μ -chain knockout NOD mice can be used to test the pathogenicity of specific autoantibodies to the loss of secretory function through selective B cell or autoantibody transfer. Since T cell help is thought to be a necessary component driving antibody generation, this model is better suited for the study of B cell contributions to disease than is the NOD-*scid* mouse. Whether sialadenitis or secretory dysfunction occurs in μ -chain knockout NOD mice is not yet known, however, the study of this strain should provide exciting new insights to the effector mechanisms of secretory dysfunction in the NOD mouse.

Perhaps the most intriguing aspect of the work in this dissertation is the finding of glandular changes in the absence of lymphocytic infiltration. These changes include morphological abnormalities, changes in tissue specific gene expression, changes in secretory proteins, increased apoptotic protease activity, and novel matrix-metalloproteinase activity. Moreover, these changes occur at the time in which

lymphocytes infiltrate the exocrine tissues and destroy glandular function. It is generally believed that histological and biochemical changes taking place in the salivary and lacrimal glands of Sjögren's Syndrome patients and animal models is a result of autoimmune activity. These data strongly suggest, however, that the development of autoimmunity in these tissues may actually be a result of underlying physiological and biochemical changes within the target tissue itself. Therefore, the target tissue organs of individuals predisposed to autoimmunity may be actively responsible for triggering or enhancing the autoimmune response.

While these tissue abnormalities are intrinsic in the NOD genetic background, similar or exacerbating tissue changes precipitated by viral infection may explain how environmental factors contribute to autoimmune development in predisposed individuals. This theory is intriguing in that, while no specific virus has been implicated in Sjögren's Syndrome, evidence of viral infection or activity is often detected in autoimmune sites. This suggests that no single virus or environmental agent will be found to precipitate autoimmune development. Instead, any number of environmental agents which promote a similar array of glandular changes could lead to autoimmune development in susceptible individuals. This provides an interesting new strategy for research in the field of autoimmunity in that the study of critical differences in glandular homeostasis could be as important to our understanding of autoimmunity as the study of immunological abnormalities.

Since exocrine gland abnormalities encompassed such a wide range of cellular processes, it would be difficult to isolate a single event which underlies these physiological changes. Instead, it appears that the entire balance of exocrine homeostasis is disturbed in

aging NOD mice. While the work in this dissertation serves mainly to outline the presence of unique glandular changes, it also uncovers several clues as to how these processes may occur. One such clue is the re-expression of PSP in the submandibular glands. During submandibular gland development, PSP is expressed until 5 days of age by a transition progenitor cell type which spawns mature submandibular acini. The re-expression of PSP in aging NOD mice suggests a potential dedifferentiation of the submandibular acini. This may additionally explain the altered and disorganized appearance of acinar tissue as seen in histological sections of aging NOD and NOD-*scid* mice. Future studies will be necessary to determine whether other characteristics of these cell types are similar as well.

 While acinar cell loss was expected in NOD mice, the finding of acinar cell loss in NOD-*scid* mice was surprising. The finding of increased cysteine protease activity in NOD-*scid* mice was similarly unexpected. While direct detection of acinar cell apoptosis in NOD-*scid* tissues using histological assays has not yet been examined, increased cysteine protease activity is highly suggestive that apoptotic cell death is taking place. Since apoptosis is considered an active, energy-driven process, detection of apoptotic activity in NOD-*scid* mice indicates that non-lymphocytic components are driving submandibular acinar cell death. While this does not discount a potential role of natural killer or antigen presenting cells in this process, it is intriguing to speculate that the actual apoptotic trigger may reside in the tissue itself. Therefore, future studies need to focus on the mechanisms leading to apoptotic cell death in NOD-*scid* exocrine tissues.

Both NOD and NOD-*scid* saliva and salivary gland lysates contain a unique matrix-metalloproteinase activity that is not present in control mice. Developmental expression of extracellular matrix components or the degradation of existing extracellular

matrix helps to drive the process of cellular differentiation and glandular remodeling in the salivary glands. It follows that, similar to dedifferentiation observed in cell culture, degeneration of the extracellular matrix in adult exocrine tissues could lead to the disruption of existing mature cell types. Therefore, it is plausible that degradation of the ECM by novel MMP activity in NOD exocrine tissues could lead to acinar cell dedifferentiation or cell death. If this hypothesis were true, it would provide a valid explanation for the extensive nature of the exocrine gland abnormalities such as novel gene transcription, proteolytic cleavage of PSP, increased acinar cell death, and ductal hyperproliferation. Future studies need to focus proteolytic activity in both NOD and normal control strains, since the absence of necessary MMP activity may be as disruptive to glandular homeostasis as the expression of novel MMP activity.

It has been speculated that protein alterations such as proteolytic cleavage could expose novel, immunogenic self-peptides to the immune system. In this way, thymic tolerance would not eliminate the generation of a self-response against this type of "cryptic" antigen. Aberrant gene expression and proteolytic cleavage of PSP in the submandibular glands, and the presence of this protein in the lacrimal gland as well, raises the possibility that PSP may represent a putative autoantigen. PSP has yet to be found in human exocrine tissues, and difficulties in obtaining and expressing large quantities of purified PSP have left this question unanswered. However, the loss of tolerance to a protein antigen does not necessarily lead to the loss of glandular tolerance. Moreover, the extensive abnormalities in the exocrine tissues of NOD mice and the unique proteolytic activity would indicate that, similar to diabetes in these mice, numerous putative autoantigens would be present. In this regard, this work has largely focused on the use of

PSP as a marker of glandular changes in NOD tissues. Future studies will investigate the specific proteases involved in PSP cleavage as well as investigate a potential role of PSP as an autoantigen.

Lastly, a promising future lies ahead for the application of congenic NOD strains used to study diabetes susceptibility intervals to the study of autoimmune sicca syndrome. Recent research in our group has shown secretory dysfunction in NOD.B10H-2^b mice which do not develop insulinitis or diabetes. This work has allowed for the development of the first proposed murine model for primary Sjögren's Syndrome. Additionally, NOD mice containing the B10 PSP gene contained on the *idd-13* gene interval will be studied to determine whether submandibular gland expression of PSP is due to differences in the NOD PSP gene itself, or caused by additional factors. These studies, as well as those described above, will serve to provide a comprehensive analysis of the mechanisms leading to secretory dysfunction in the NOD mouse, with future implications for our long-term goal of understanding the pathogenesis of human Sjögren's Syndrome.

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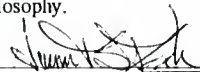
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BIOGRAPHICAL SKETCH

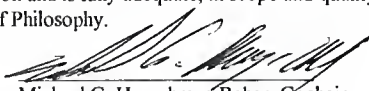
Christopher Paul Robinson was born in Northampton, MA., on March 25, 1970, the youngest of three children born to Paul and Wesley Robinson. He completed his undergraduate education in molecular biology at Lehigh University in 1992. In January of 1993, he moved to Gainesville, FL, to begin working toward his Ph.D. at the University of Florida, Department of Pathology and Laboratory Medicine under the mentorship of Dr. Ammon Peck. His research goals led him to undertake a joint project with the Department of Oral Biology with the comentorship of Dr. Michael Humphreys-Beher. On June 19, 1994, Chris married his college sweetheart, Meryl Weiss, in Woodbury, NY.

To date, he has authored five articles which have been submitted or published in peer reviewed journals, and has appeared as a coauthor on several additional publications. In addition, he has presented his findings at the 5th International Meeting on Sjögren's Syndrome in Amsterdam, the Second International Conference on the Lacrimal Gland, Tear Film, and Dry Eye Syndromes in Bermuda, and the 25th Annual Meeting of the American Association of Dental Research, where he received the Edward H. Hatton Award for excellence in predoctoral research. Chris plans to continue his work in the field of autoimmune Sjögren's Syndrome in collaboration with his mentors at the University of Florida and Dr. Roland Jonsson in Bergen, Norway.

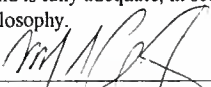
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Ammon B. Peck, Chair
Professor of Pathology and
Laboratory Medicine

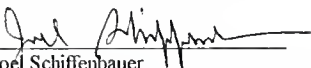
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Michael G. Humphreys-Beher, Cochair
Professor of Oral Biology

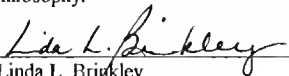
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Assistant Professor of Pathology and
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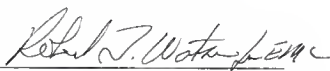

Joel Schiffenbauer
Associate Professor of Molecular Genetics
and Microbiology

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Professor of Orthodontics

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted for partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1997


Dean, College of Medicine


Dean, Graduate School

